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Jean-Philippe RASIGADE

Rôle physiopathologique de l'internalisation de *Staphylococcus aureus* par les ostéoblastes au cours de l'infection osseuse

Directeur de thèse : Docteur Frédéric LAURENT

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Professeur Laurent MEREGHETTI, Rapporteur
Professeur Anne-Claude CREMIEUX
Professeur Jérôme ETIENNE
Professeur François VANDENESCH
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Rôle physiopathologique de l'internalisation de *Staphylococcus aureus* par les ostéoblastes au cours de l'infection osseuse

Résumé

L'invasion des ostéoblastes par *Staphylococcus aureus* (*SA*) est considérée comme responsable, au moins partiellement, de l'évolution chronique ou récurrente des infections osseuses (IO). Nous avons émis l'hypothèse que des différences d'interactions *SA*-ostéoblastes pouvaient être associées aux différences de présentation clinique des IO. Nous avons d'abord développé un modèle ex vivo d'infection intracellulaire d'ostéoblastes humains permettant de quantifier l'adhésion, l'invasion, la survie intracellulaire de *SA* et les dommages subis par les cellules infectées. Grâce ce modèle, nous avons montré que les *SA* communautaires résistants à la méticilline (CA-MRSA), un groupe polyphylétique de souches hypervirulentes associées à des formes aiguës et sévères d'IO, induisent une cytotoxicité supérieure à celle des MRSA hospitaliers (HA-MRSA) associés à des IO plus souvent chroniques. A l'aide de mutants isogéniques, nous avons pu démontrer que cette cytotoxicité était indépendante de la toxine de Panton-Valentine et l'alpha-hémolysine mais associée à la surexpression des phenol-soluble modulins (PSM) par les CA-MRSA. Ces résultats ont permis d'identifier un nouveau mécanisme de virulence des CA-MRSA basé sur l'invasion des ostéoblastes et l'activité intracellulaire des PSM. Parallèlement, nous avons montré que certains antibiotiques modifient le niveau de transcription et d'expression des protéines staphylococciques impliquées dans l'invasion des ostéoblastes, sans que nous ne puissions montrer une modification de la capacité d'invasion de *S. aureus* dans ce même modèle ex vivo. Nos travaux ouvrent de nouvelles perspectives dans la compréhension et la prise en charge des IO due à *SA*.

Mots-clef français

Staphylococcus aureus ; infection osseuse ; pathogénie bactérienne ; toxines staphylococciques ; invasion bactérienne ; survie intracellulaire ; phenol-soluble modulins ; leucocidine de Panton-Valentine ; alpha-toxine ; fibronectine ; MRSA ; CA-MRSA ; HA-MRSA

Role of osteoblast invasion by *Staphylococcus aureus* in the pathogenesis of osteomyelitis

Summary

Osteoblast invasion by *Staphylococcus aureus* (SA) is currently considered a putative explanatory mechanism for the chronic or recurrent nature of osteomyelitis. We raised the hypothesis that inter-strain differences in the interactions between *S. aureus* and osteoblasts at the cellular level could correlate with differences in the clinical presentation of osteomyelitis. We first developed an ex vivo model of intracellular bacterial challenge of human osteoblasts to quantify SA adhesion, invasion and intracellular survival as well as SA-induced damage to infected cells. By means of this model, we have demonstrated that community-acquired methicillin-resistant SA (CA-MRSA) strains, which belong to a polyphyletic group endowed with high virulence and are associated with severe and acute forms of osteomyelitis, induce more cytotoxicity in osteoblasts as compared to hospital MRSA strains, which in turn are more frequently involved in chronic forms of osteomyelitis. Using isogenic CA-MRSA mutants, we determined that SA-induced osteoblast damage was independent of the production of Panton-Valentin leukocidin and alpha-toxin, but was associated with the overexpression of phenol-soluble modulins (PSMs) by CA-MRSA. These findings elucidate a novel virulence strategy of CA-MRSA based on the invasion and PSM-related killing of osteoblasts. In parallel to this research, we demonstrated that several antibiotics alter the transcription and expression levels of SA adhesins involved in osteoblast invasion. However, antibiotics did not induce changes in SA invasiveness in our ex vivo infection model. Collectively, our findings provide new insights into the pathogenesis of SA osteomyelitis.

Keywords

Staphylococcus aureus; osteomyelitis; bacterial pathogenesis; staphylococcal toxins; bacterial invasion; intracellular survival; phenol-soluble modulins; Panton-Valentine leukocidin; alpha-toxin; fibronectin; MRSA; CA-MRSA; HA-MRSA

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Adresse postale du laboratoire :

INSERM U851
Faculté de Médecine Lyon-Est
7 rue Guillaume Paradin
69372 LYON CEDEX 8 – France

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ABREVIATIONS

agr	*Accessory gene regulator*
CA-MRSA	*S. aureus* résistant à la méticilline communautaire
CFTR	Cystic fibrosis transmembrane conductance regulator
CMI	Concentration minimale inhibitrice
FAK	Focal adhesion kinase
FnBP	Fibronectin-binding protein
FPR2	Formyl peptide receptor 2
HA-MRSA	*S. aureus* résistant à la méticilline hospitalier
IL	Interleukine
ILK	Integrin-linked kinase
IOA	Infection ostéo-articulaire
LAC	Los Angeles County (origine de la souche prototype LAC USA300)
LukF-PV	Composant F (fast-eluted) de la PVL
LukS-PV	Composant S (slow-eluted) de la PVL
MOI	Multiplicity of infection
MRSA	*S. aureus* résistant à la méticilline
MSSA	*S. aureus* sensible à la méticilline
NLR	Nod-like receptor
ORF	Open reading frame, cadre de lecture
PFT	Pore-forming toxin
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
PVL	Leucocidine de Panton-Valentine
RANK	Receptor activator for NFkB
RANKL	RANK-Ligand
sae	*Staphylococcus aureus* exoprotein expression regulator
sar	Staphylococcal accessory regulator
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCN	Staphylocoque à coagulase négative
SCV	Small colony variant
ST	Sequence type
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-receptor associated factor
TRAIL	TNF apoptosis-inducing ligand
TRAP	Tartrate-resistant acid phosphatase

TRAVAIL BIBLIOGRAPHIQUE

I. INTRODUCTION

Les infections ostéo-articulaires (IOA) regroupent un ensemble d'entités cliniques ayant en commun l'invasion et la destruction progressive des tissus osseux et cartilagineux par des micro-organismes, le plus souvent des bactéries. Le taux de mortalité associé aux IOA est faible comparativement à d'autres maladies infectieuses, cependant leur évolution est grevée d'une morbidité majeure et d'un risque élevé de séquelle fonctionnelle. Ces entités cliniques présentent des caractéristiques physiopathologiques distinctes permettant leur classification en fonction de leur localisation, articulaire ou osseuse, et de la source de l'infection, qui peut être hématogène ou par contiguïté (Lew et Waldvogel, 2004). Ces infections peuvent être aiguës ou chroniques, peuvent concerner des articulations natives, particulièrement la hanche et la cheville, des articulations prothétiques, ou les os longs, les vertèbres et potentiellement n'importe quel tissu osseux. Les IOA sont parmi les maladies infectieuses les plus difficiles à prendre en charge, particulièrement lorsqu'elles évoluent sur un mode chronique ou récidivant et impliquent un matériel implanté.

Le pathogène le plus souvent en cause dans les IOA est *Staphylococcus aureus* ; cette bactérie présente un remarquable pouvoir d'adaptation, et a pu développer des mécanismes de virulence et de résistance variés. En particulier, l'émergence récente et la diffusion explosive dans certaines régions du monde de souches de *S. aureus* présentant à la fois une résistance aux antibiotique et une virulence accrue, à l'origine d'IOA aiguës et sévères, a mis en lumière l'importance sans cesse renouvelée de ce pathogène et le problème de santé publique qu'il

représente. L'implication de *S. aureus* dans les infections du site opératoire en orthopédie fait également peser un lourd fardeau sur les sytèmes de santé, dont le poids futur ira probablement croissant au même rythme que l'augmentation du nombre d'arthroplasties pratiquées si l'on considère que le taux d'attaque des infections sur prothèse reste stable, autour de 1 à 2% (Zimmerli *et al.*, 2004) : 220 000 prothèses de hanche ont été posées aux Etats-Unis en 2003, ce qui représentait une hausse de 38% par-rapport aux données de 1996. Lee et Goodman ont estimé que ce chiffre atteindrait 572 000 en 2030 (Lee et Goodman, 2008). De la même façon, le nombre annuel d'arthroplasties totales du genou, qui était de 418 000 en 2003, est amené à augmenter rapidement dans un contexte de vieillissement de la population dans les pays à hauts revenus.

Au cours des arthrites septiques, la prise en charge médicale par antibiothérapie est le plus souvent efficace à condition d'être initiée rapidement, d'être documentée microbiologiquement et d'être accompagnée d'un drainage (Shirtliff et Mader, 2002). Inversement, l'infection osseuse est souvent réfractaire à l'antibiothérapie, et ce problème est exacerbé par les taux croissants d'antibiorésistance chez *Staphylococcus* spp. Enfin, une difficulté supplémentaire surgit avec l'émergence possible in situ de formes persistantes et résistantes de *small colony variants*, potentiellement sélectionnées par certaines antibiothérapies (Von Eiff *et al.*, 1997; Ciampolini et Harding, 2000; Henderson et Nair, 2003; Von Eiff *et al.*, 2006). Les IOA staphylococciques constituent donc et demeureront dans les années à venir un problème majeur en infectiologie. Dans ce contexte, une meilleure compréhension des interactions entre ces pathogènes et le tissu osseux est cruciale pour l'optimisation de la prise en charge des patients, voire le développement de stratégies thérapeutiques nouvelles.

Au cours de ce travail bibliographique, nous exposerons la physiopathologie générale des IOA à *S. aureus*, puis nous décrirons spécifiquement les relations hôte-pathogène considérées comme pertinentes pour notre compréhension des difficultés de traitement propres aux IOA, en mettant l'accent sur l'invasion des cellules osseuses par *S. aureus* et ses conséquences. Nous nous intéresserons enfin au cas particulier des souches résistantes et hypervirulentes connues sous le nom de *community-acquired methicillin-resistant S. aureus* (CA-MRSA), dont la diffusion

pandémique a profondément modifié le pronostic des IOA dans certains pays, notamment les Etats-Unis.

II. LES INFECTIONS OSTEO-ARTICULAIRES

Les IOA regroupent d'une part les infections du tissu osseux proprement dit, et d'autre part les arthrites septiques qui présentent certaines particularités physiopathologiques. Au cours de ce travail, nous nous focaliserons sur la physiopathologie des infections osseuses et présenterons plus brièvement les arthrites septiques.

II.A. Les infections osseuses

II.A.1. *Les infections osseuses hématogènes*

Dans le vocabulaire français, l'ostéomyélite désigne classiquement une infection osseuse d'origine hématogène sans corps étranger. Cette terminologie peut être source de confusion car le terme « osteomyelitis » dans la littérature anglo-saxonne désigne l'ensemble des infections osseuses. Dans la suite de cette thèse, le terme « ostéomyélite » sera employé dans son acception francophone.

L'ostéomyélite est déclenchée par l'inoculation du tissu osseux par une bactérie issue de la circulation sanguine. Dans l'ostéomyélite primaire, cette bactériémie initiale est asymptomatique et l'infection osseuse est au premier plan des signes infectieux. Bien qu'elle soit présente chez l'adulte, cette forme clinique prédomine largement chez l'enfant (Lew et Waldvogel, 2004). Les ostéomyélites de l'adulte sont plus fréquemment secondaires à un foyer infectieux annexe préexistant comme une endocardite, source de la bactériémie. Les sites anatomiques les plus communément touchés sont les corps vertébraux (spondylodiscite) et les os longs. La forme typique d'ostéomyélite primaire des os longs implique une lésion unique de la partie distale du tibia proche de la métaphyse (Carek *et al.*, 2001). Cette

implication fréquente de la métaphyse est expliquée par l'anatomie particulière du tissu osseux dans cette zone. Le flux sanguin est à cet endroit ralenti et turbulent, ce qui favorise l'adhésion et la colonisation de l'endothélium par les bactéries circulantes. Un antécédent récent de traumatisme, même mineur, favorise également l'infection chez l'enfant en lien avec un hématome local, une obstruction vasculaire et/ou une micro-zone nécrotique très sensible à l'inoculation bactérienne (Morrissy et Haynes, 1989). L'inflammation locale déclenchée par l'infection provoque alors un afflux de leucocytes et une chute du pH et de l'oxygénation, autant de phénomènes qui compromettent à leur tour la circulation médullaire et favorisent l'extension de l'infection. Chez le nourrisson, la présence de vaisseaux sanguins dans la plaque de croissance épiphysaire peut également permettre l'extension de l'infection à l'articulation depuis un point de départ métaphysaire ; cependant, après 1 an de vie cette plaque est dévascularisée et l'infection reste confinée à la métaphyse et à la diaphyse.

L'évolution chronique d'une ostéomyélite est associée à la formation de plages de nécrose osseuse, les séquestres (Figure 1) (Lew et Waldvogel, 2004). Ces fragments de tissu dévascularisés et détachés du tissu avoisinant sont inaccessibles aux cellules immunitaires et aux antibiotiques et se comportent comme un corps étranger inerte vis-à-vis de l'adhésion, de la colonisation bactérienne et de la formation de biofilm. Dans les formes non traitées d'ostéomyélite chronique, devenues rares dans les pays industrialisés, les séquestres osseux peuvent avoir deux destins déterminés par leur taille. Les séquestres de petite taille sont progressivement résorbés par un tissu de granulation recruté par les signaux inflammatoires issus de la zone nécrotique. Lorsque l'étendue des séquestres est trop importante ou que la réponse inflammatoire de l'hôte est compromise, leur extension est progressivement confinée par une néoformation osseuse issue du périoste, décollé de la corticale osseuse par l'accumulation de pus qui peut également se fistuliser à la peau (Figure 2) (Hankin *et al.*, 2011). Cette néoformation, l'involucre, permet d'assurer la continuité de l'os et le maintien a minima de sa fonction pendant la phase de convalescence. Cependant, la vascularisation inadéquate des tissus circonscrits par l'involucre favorise le maintien des séquestres osseux sous-jacents, aboutissant à une pathologie chronique dans laquelle le débridement chirurgical est souvent la seule option.

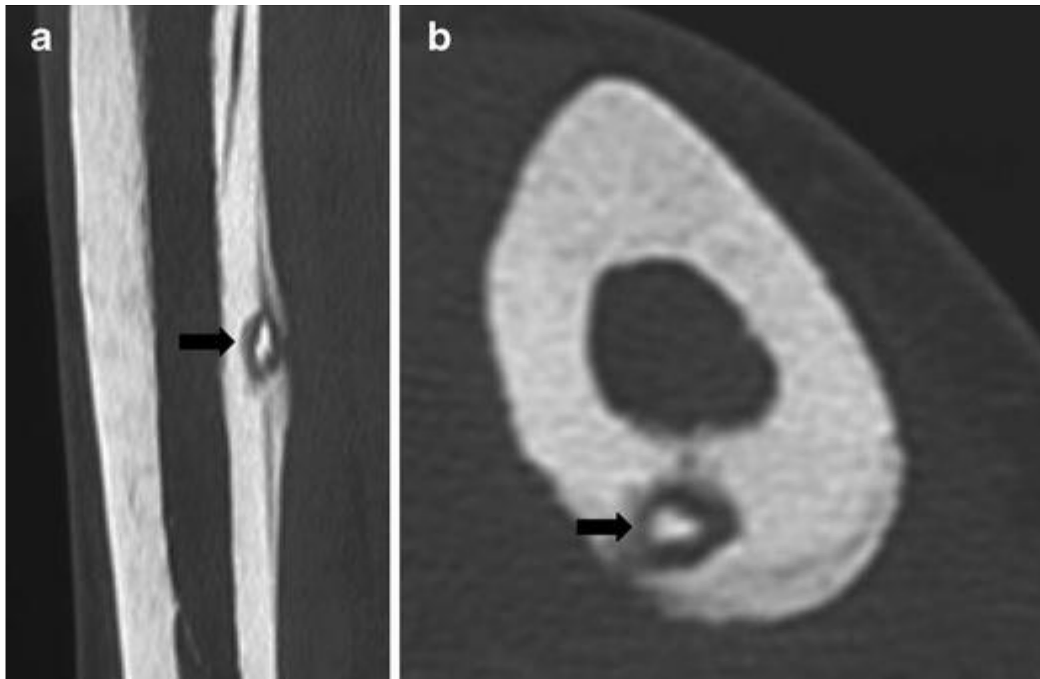

Figure 1. Séquestre intra-cortical du tibia droit chez un homme de 21 ans. Coupes coronale (a) et tranverse (b) montrant une image abcédée comprenant en son centre une zone dense calcifiée (flèche). D'après (Jennin *et al.*, 2010).

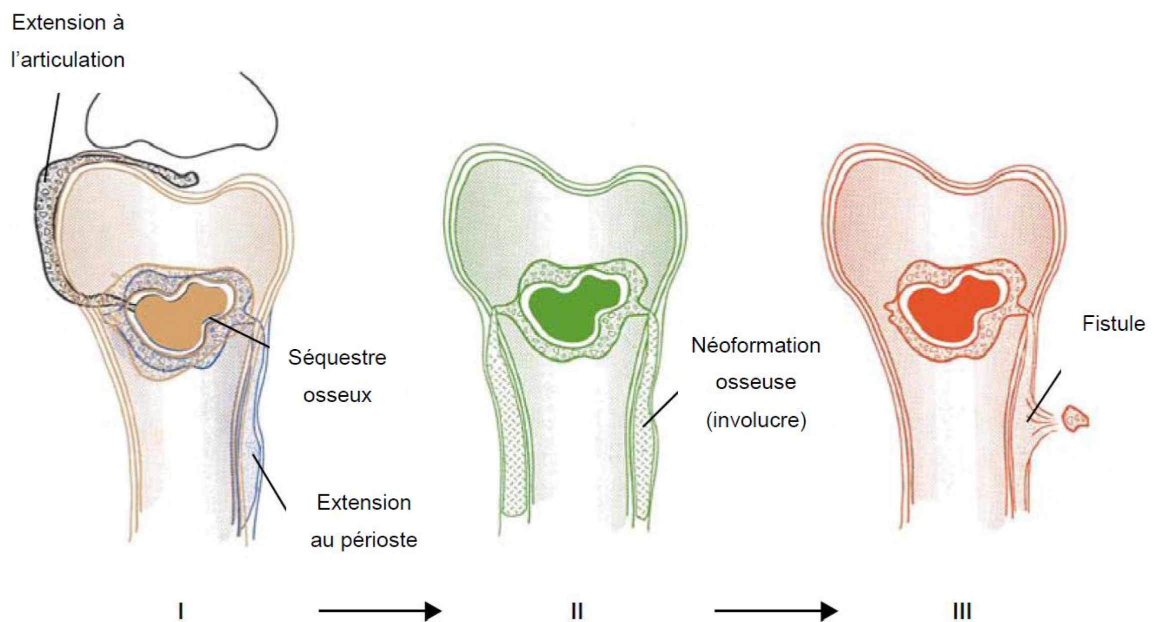

Figure 2. Progression de l'ostéomyélite chronique. Phase I : depuis un séquestre constitué, la progression de l'infection intramédullaire vers l'intérieur de la capsule articulaire peut aboutir à une arthrite septique ; la progression de l'infection vers la zone périostée peut aboutir à la constitution d'un abcès périosté. Phase II : la persistance d'un décollement du périoste conduit à la constitution d'une néoformation osseuse, l'involucre. Phase III : l'extension de l'infection à travers l'os cortical et le périoste forme un trajet fistuleux pouvant s'aboucher à la peau. D'après (Lew et Waldvogel, 2004).

Chez l'enfant, l'incidence des ostéomyélites hématogènes est classiquement entre 1 et 2 cas pour 100 000 (Weichert *et al.*, 2008). L'incidence des ostéomyélites en pédiatrie semble décliner lentement, avec une baisse annuelle de 0.185 cas pour 100 000 enregistrée à Glasgow, R-U, entre 1970 et 1997 (Blyth *et al.*, 2001; Lazzarini *et al.*, 2004; Weichert *et al.*, 2008).

II.A.2. *Les infections osseuses par contiguïté*

Les infections osseuses par contiguïté sont plus fréquentes que les ostéomyélites, et cette tendance s'est accentuée depuis les années 1980 (Espersen *et al.*, 1991). Leur point de départ peut être une infection des parties molles adjacentes (notamment chez le patient diabétique ou à la suite d'un escarre de décubitus), ou l'inoculation directe de bactéries au site de l'infection consécutive à un traumatisme ou lors d'un abord chirurgical avec rupture d'aseptie. La physiopathologie des IOA par contiguïté présente certains points communs avec les ostéomyélites. En particulier, la présence d'un traumatisme interfère avec la vascularisation tissulaire locale et crée des plages nécrotiques qui favorisent la colonisation bactérienne.

L'incidence des infections osseuses par contiguïté, contrairement à celle des ostéomyélites, est en augmentation (Gillespie, 1990; Lazzarini *et al.*, 2004). Ces infections, qu'elles émergent de l'infection de tissus adjacents à l'os ou d'une inoculation directe, peuvent toucher des patients de tous âges, en particulier les porteurs d'articulation prothétique (Lew et Waldvogel, 2004). La présence d'une prothèse est particulièrement associée à l'évolution chronique de l'infection osseuse ; dans ce contexte, la prise en charge médicale par antibiothérapie est fréquemment mise en échec, et la dépose de la prothèse, accompagnée d'un débridement chirurgical, devient nécessaire (Ciampolini et Harding, 2000; Trampuz et Zimmerli, 2008). Des cas d'infection osseuse récurrentes dont les épisodes sont espacée de plusieurs années ont été documentés, ainsi que des cas de rechute survenant 50, voire 80 ans après l'infection initiale (Gallie, 1951; Korovessis *et al.*, 1991; Greer et Rosenberg, 1993; Ciampolini et Harding, 2000).

II.B. L'arthrite septique

L'arthrite septique est associée à la colonisation bactérienne d'une articulation (Levine et Siegel, 2003). L'infiltration et le développement bactérien dans la synovie déclenche une réponse inflammatoire et le recrutement de leucocytes dans le liquide articulaire (Goldenberg, 1998; Nade, 2003). La production locale de radicaux libres, ou espèces réactives de l'oxygène, de métalloprotéases, d'enzymes lysosomales et de toxines bactériennes contribue à la destruction du cartilage. La dégradation des protéoglycanes et du collagène de l'hôte est une conséquence de l'afflux de polynucléaires et survient dès les étapes initiales de l'infection (Goldenberg, 1998; Stott, 2001; Shirtliff et Mader, 2002; Nade, 2003). Une spécificité physiologique de l'arthrite septique est que ce processus inflammatoire prend place dans un environnement inextensible, délimité par la capsule articulaire ; l'inflammation provoque ainsi une augmentation de pression intra-articulaire, responsable à son tour d'une diminution de vascularisation et d'une exacerbation de la destruction du cartilage et de la synovie. L'ensemble de ces processus peut conduire rapidement à une destruction définitive du cartilage et de l'os sous-chondral, en quelques jours d'évolution (Shirtliff et Mader, 2002).

L'incidence de l'arthirte septique se situe entre 2 et 10 pour 100 000 patients dans la population générale, mais peut atteindre 30 à 70 pour 100 000 chez les patients présentant des facteurs de risque comme la polyarthrite rhumatoïde ou la présence de matériel prothétique (Goldenberg, 1998; Stott, 2001; Nade, 2003). L'arthrite septique est également plus fréquente chez les enfants que chez les adultes, et chez les hommes que chez les femmes (Levine et Siegel, 2003).

II.C. Epidémiologie bactérienne des IOA

De nombreuses espèces bactériennes ont été isolées de cas d'arthrite septique et d'infection osseuse. Au cours d'arthrites septiques ont été incriminés *S. aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens* ainsi que les genres *Salmonella*, *Neisseria*, *Aerobacter* et *Bacteroïdes* (Shirtliff et Mader, 2002; Nade, 2003). Les pathogènes responsables d'infections osseuses comprennent les genres *Staphylococcus*, *Streptococcus*, *Salmonella*, *Mycobacterium* ainsi qu'*E.coli*, *P. aeruginosa* et *Haemophilus influenzae* (Lew et Waldvogel, 1997; Lazzarini *et al.*, 2004; Lew et Waldvogel, 2004). Cependant, *S. aureus* est de loin le pathogène le plus fréquemment en cause, autant dans l'arthrite septique que dans l'infection osseuse, et ce quelle que soit la voie de contamination (Goldenberg, 1998; Ciampolini et Harding, 2000; Lew et Waldvogel, 2004).

Le genre *Staphylococcus*, principalement *S. aureus*, est responsable de 37 à 67% des cas d'arthrite septique dans des populations d'origine géographiques variées (Ryan *et al.*, 1997; Goldenberg, 1998; Dubost *et al.*, 2002; Al Arfaj, 2008). Bien que la prévalence de *Staphylococcus* spp. dans ces infections soit toujours élevée, de variations importantes sont observées en fonction du recrutement des services. Les staphylocoques à coagulase négative (SCN) sont moins fréquemment isolés d'arthrites septiques, et représentent 3 à 16% des isolats de *Staphylococcus* spp. (Ryan *et al.*, 1997; Dubost *et al.*, 2002; Al Arfaj, 2008). La prévalence des staphylocoques au cours des infections osseuses est sensiblement équivalente à celle observée dans les arthrites septiques : *S. aureus* est isolé dans 38 à 67% des cas, les staphylocoques à coagulase négative dans 5 à 15% (Karwowska *et al.*, 1998; Blyth *et al.*, 2001; Arnold *et al.*, 2006; Grammatico *et al.*, 2008). Les rapports de la Health Protection Agency du Royaume-Uni sur la surveillance des infections du site opératoire (ISO) entre 1997 et 2005 ont pu déterminer que *S. aureus* était responsable de 41.4% des cas d'infection sur prothèse totale de hanche, 59.1% des cas d'infection sur prothèse partielle de hanche, 33.5% des cas d'infection sur prothèse de genou et 53% des cas d'infection après réduction chirurgicale de fracture ouverte. Les SCN étaient responsables quant à eux de 15.1%, 6.3%, 20.7%

et 7.5% de ces infections, respectivement (U.K. Health Protection Agency, 2008). *S. epidermidis* est le SCN le plus fréquent dans de nombreux types d'infection, y compris les infections osseuses et les infections sur matériel prothétique, mais d'autres espèces de SCN ont été incriminées dont *S. simulans*, *S. hominis*, *S. capitis*, *S. caprae* et *S. lugdunensis* (Murdoch *et al.*, 1996; Lang *et al.*, 1999; Greig et Wood, 2003; Vallianou *et al.*, 2008).

III. INTERACTIONS HOTE-PATHOGENE AU COURS DES IOA

Le tissu osseux présente une physiologie complexe, et les interactions entre *S. aureus* et les différents constituants de ce tissu sont encore imparfaitement comprises. Dans ce chapitre, nous détaillerons les mécanismes physiopathologiques associés à la pathogénie particulière des IOA, sous l'angle de la perte de tissu osseux et des échecs de traitement médical. Après avoir rappelé les principales caractéristiques du tissu osseux sain, nous détaillerons : (i) les conséquences de la réponse inflammatoire déclenchée par l'infection staphylococcique au sein de ce tissu ; (ii) l'invasion des cellules osseuses, les ostéoblastes, par *S. aureus* et ses conséquences tant pour les cellules infectées que pour les bactéries intracellulaires, avec l'émergence des phénotypes dormants *small colony variants* ; et (iii) la formation des biofilms bactériens et son rôle dans la résilience bactérienne au cours des IOA. Nous concluerons ce chapitre par une brève synthèse de l'impact de chacun des mécanismes physiopathologiques abordés sur les modalités de prise en charge des IOA.

III.A. Physiologie du tissu osseux

L'os mature est constitué en périphérie d'os cortical, composé de lamelles denses de matrice minéralisée, entourant un réseau de travées osseuses désigné sous le nom d'os trabéculaire. Ces deux formes de tissu osseux sont constituées des mêmes élements cellulaires, matriciels et minéraux, mais présentent des différences de structure et de fonction. L'os cortical compact joue un rôle de résistance aux forces de cisaillement, alors que l'os trabéculaire, dont les travées sont orientées le long des lignes de charge, joue un rôle de résistance à la compression. La surface

de l’os trabéculaire représente environ 80% de l’interface os-moelle. Le squelette dans son ensemble est un système dynamique, dans un état de renouvellement continu assuré par l’action de deux types cellulaires, les ostéoblastes et les ostéoclastes. Chez l’adulte, l’os trabéculaire est physiologiquement renouvelé tous les 3 ou 4 ans, alors que la durée de turn-over de l’os cortical est de plus de 10 ans (Blair, 1998). Ce remodelage continu est nécessaire pour assurer le remplacement des tissus anciens, la réparation des micro-fractures et ainsi assurer l’intégrité du tissu osseux et l’homéostasie de sa matrice (Vaananen et Laitala-Leinonen, 2008).

III.A.1. ***Ostéoblastes et ostéocytes***

Les ostéoblastes assurent la synthèse de la matrice osseuse ; ils sont localisés sur les surfaces osseuses et dérivent de progéniteurs mésenchymateux. Ces cellules sécrètent l’ostéoïde, composé de protéines matricielles dont plus de 90% de collagène de type I, de protéoglycanes comme la décorine et le biglycane, de glycoprotéines dont la fibronectine et l’ostéonectine, d’ostéopontine, d’ostéocalcine et de sialoprotéine osseuse, ces protéines étant orientées le long des lignes de stress (Figure 3) (Blair, 1998; Mackie, 2003). Les ostéoblastes participent également à la minéralisation de la matrice osseuse par le dépôt d’hydroxyapatite (Blair, 1998; Mackie, 2003). Les mécanismes impliqués dans la minéralisation osseuse sont encore imparfaitement connus. Il semble que des facteurs déclencheurs, appelés facteurs de nucléation, soient également nécessaires pour initier la minéralisation ; les protéines phosphatées comme la sialoprotéine osseuse ou l’ostéopontine jouent probablement ce rôle (Henderson et Nair, 2003; Mackie, 2003; Huitema et Vaandrager, 2007). Les ostéoblastes synthétisent également une phosphatase alcaline capable de cliver les esters de phosphate, libérant ainsi le phosphate libre inorganique nécessaire au processus de minéralisation (Huitema et Vaandrager, 2007).

Le stade « ostéoblaste » n’est pas une phase terminale de différentiation. Les ostéoblastes qui n’entrent pas en apoptose évoluent en ostéocytes après leur inclusion dans la matrice osseuse, qu’ils cessent de produire (Blair, 1998; Mackie,

2003). Les ostéocytes sont impliqués dans l'entretien du tissu osseux, et jouent un rôle de détection du stress mécanique à l'aide de projections cellulaires étendues, les canalicules, qui interconnectent les ostéocytes entre eux et sont sensibles aux contraintes mécaniques (Mackie, 2003; Huitema et Vaandrager, 2007). Les ostéocytes répondent au stress mécanique en entrant en apoptose, avec pour conséquence le recrutement local et la différentiation d'ostéoclastes, probablement médiés par la modification des concentrations en facteurs diffusibles sécrétés par l'ostéocyte. Un de ces facteurs pourrait être le *transforming growth factor* β (TGF-β), qui supprimerait l'ostéoclastogénèse lorsqu'il est sécrété par des ostéocytes viables (Matsuo et Irie, 2008; Henriksen *et al.*, 2009).

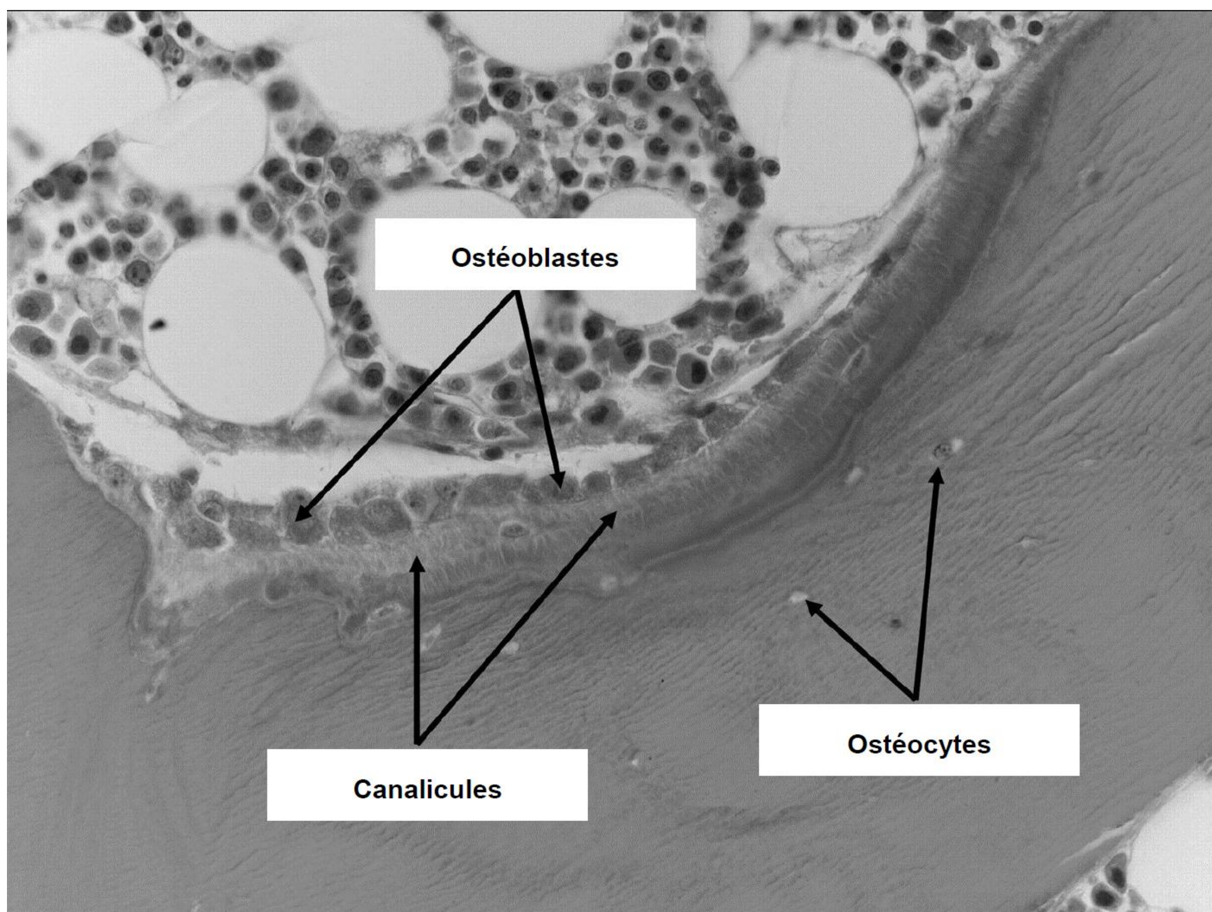

Figure 3. Synthèse de la matrice osseuse par les ostéoblastes. Les ostéoblastes synthétisent une matrice protéique, l'ostéoïde, principalement constituée de collagène de type I. Cette matrice protéique est ensuite minéralisée pour former le tissu osseux mature. Au fur et à mesure de la progression de la formation de matrice osseuse, les ostéoblastes qui ne rentrent pas en apoptose sont emprisonnés dans l'os minéralisé et atteignent leur stade terminal de différentiation sous forme d'ostéocytes. D'après (Clarke, 2008).

III.A.2. Ostéoclastes

L'action de résorption de la matrice osseuse est assurée par des cellules multinuclées, les ostéoclastes. Contrairement aux ostéoblastes d'origine mésenchymateuse, les ostéoclastes dérivent de la lignée myéloïde (Figure 4). La résorption du tissu osseux, constitué de protéines enchassées dans une matrice minéralisée, peut se décomposer en deux étapes : (i) la déminéralisation de la matrice, permettant l'accès aux constituants protéiques ; et (ii) la dégradation par protéolyse des constituants protéiques.

Les ostéoclastes expriment à leur surface des H^+-ATPases ainsi que des canaux chloriques, permettant la sécrétion d'acide chlorhydrique dans un compartiment fermé, la lacune de résorption (Blair *et al.*, 1989; Blair, 1998; Kornak *et al.*, 2001; Vaananen et Laitala-Leinonen, 2008). La lacune de résorption est étanchéifiée par une membrane périphérique par laquelle l'ostéoclaste adhère à la matrice osseuse ; conjointement au transport transmembranaire d'ions H^+, la fusion de vésicules acidifiées avec la membrane plasmique contribue à la sécrétion acide (Vaananen et Laitala-Leinonen, 2008). Cette acidification locale permet la solubilisation de la fraction minérale de la matrice osseuse, qui rend alors possible la protéolyse des constituants protéiques. Un acteur central de cette protéolyse est la cathepsine K, exprimée à haut niveau par les ostéoclastes et capable de lyser le collagène et l'ostéonectine (Bossard *et al.*, 1996; Drake *et al.*, 1996). Les ostéoclastes sécrètent également des phosphatases acides, dont la *tartrate-resistant acid phosphatase* (TRAP), activée après clivage par la cathepsine K et qui peut être utilisée comme marqueur de l'activité ostéoclastique (Blair *et al.*, 1989; Blair, 1998; Ljusberg *et al.*, 2005). Bien que la fonction exacte de TRAP dans la résorption osseuse ne soit pas élucidée, son implication dans ce phénomène est fortement suggérée par le fait que : (i) TRAP est spécifique des ostéoclastes ; (ii) son niveau sérique est corrélé à l'activité de résorption ; et (iii) des souris knock-out pour TRAP présentent un défaut de résorption osseuse et un excès de minéralisation (Hayman *et al.*, 1996; Nesbitt et Horton, 1997), alors que des souris surexprimant TRAP présentent une accélération du renouvellement osseux (Salo *et al.*, 1997; Angel *et al.*, 2000).

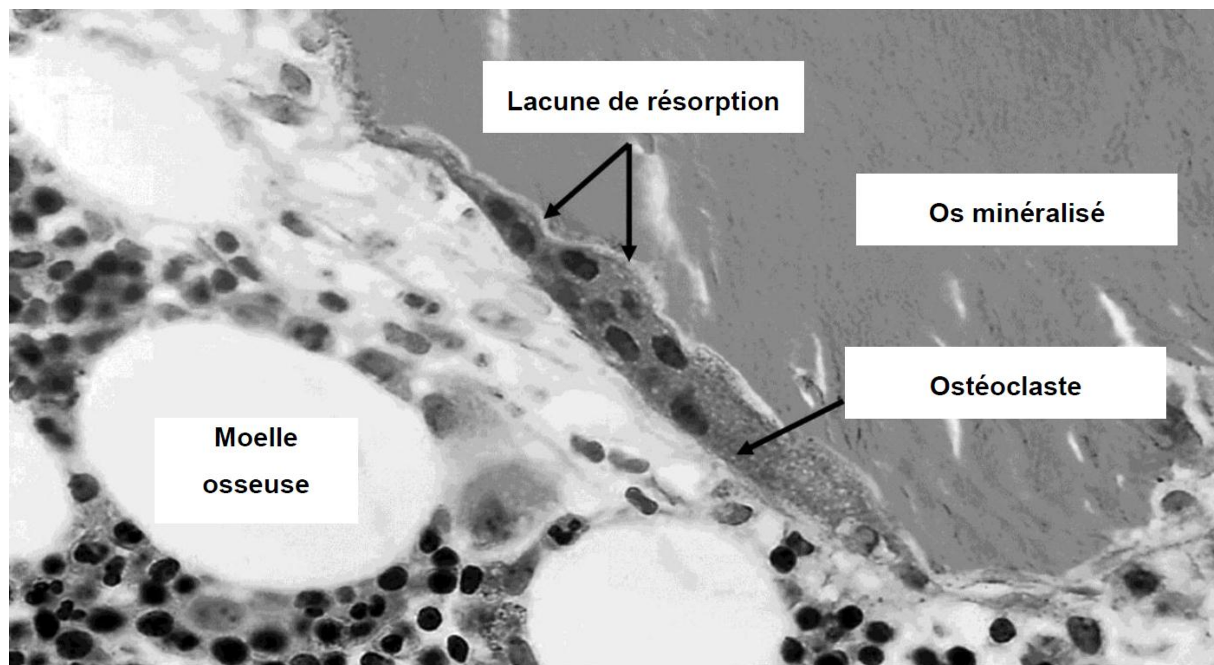

Figure 4. Ostéoclaste multinucléé et sa lacune de résorption. D'après (Clarke, 2008).

III.A.3. Régulation du remodelage osseux par les ostéoblastes et les ostéoclastes

L'équilibre de l'activité de ces deux types cellulaires, ostéoblastes et ostéoclastes, est indispensable au maintien de l'homéostasie osseuse et à son renouvellement. Tout déséquilibre dans leurs niveaux relatifs d'activité peut induire une pathologie osseuse (Henderson et Nair, 2003). L'infection par un pathogène comme *S. aureus* peut être à l'origine d'un tel déséquilibre, médié en partie par la réponse inflammatoire. Il existe une interaction permanente entre ostéoblastes et ostéoclastes, les premiers étant capables d'interpréter différents signaux de danger et de moduler en retour la différentiation et l'activité des seconds (Henderson et Nair, 2003; Matsuo et Irie, 2008). Une interaction essentielle dans la régulation de l'ostéoclastogénèse est celle qui existe entre le *receptor activator for nuclear factor* κB (RANK), exprimé par les précurseurs ostéoclastiques, et son ligand RANKL exprimé par les ostéoblastes (Figure 5) (Matsuo et Irie, 2008). RANKL est une protéine homotrimérique exprimée à la surface des ostéoblastes ou sécrétée après clivage par les *matrix metalloproteinase* (MMP) 7 et 14 ou par les protéases de la

famille *a disintegrin and metalloprotease domain* (ADAM) (Lynch *et al.*, 2005; Hikita *et al.*, 2006; Boyce et Xing, 2008). Le récepteur RANK est une protéine transmembranaire homotrimérique qui appartient à la superfamille des récepteurs du *tumor necrosis factor* (TNF). La liaison RANK-RANKL provoque le recrutement des protéines de signalisation de la classe des *TNF receptor-associated factors* (TRAF), en particulier TRAF5 et TRAF6 (Kim *et al.*, 1999; Wada *et al.*, 2006). TRAF6 semble être un acteur majeur dans l'ostéoclastogénèse médiée par RANK, comme le montre le fait que des souris knock-out pour TRAF6 développent une ostéopétrose (Lomaga *et al.*, 1999). TRAF5 semble jouer un rôle plus marginal (Kanazawa *et al.*, 2003). La signalisation médiée par RANK et TRAF6 active plusieurs facteurs de transcription dont NFκB, aboutissant à la différentiation ostéoclastique (Wada *et al.*, 2006; Matsuo et Irie, 2008). Un autre acteur majeur de la relation RANK-RANKL est l'ostéoprotégérine (OPG) (Figure 5). Cet inhibiteur endogène du signal RANKL est synthétisé par les ostéoblastes et fonctionne comme un récepteur antagoniste, capable de fixer RANKL et d'empêcher sa liaison avec RANK (Yasuda *et al.*, 1998; Theoleyre *et al.*, 2004; Wada *et al.*, 2006; Boyce et Xing, 2008). Au final, les ostéoblastes assurent la modulation de l'activité ostéoclastique via la balance d'expression de RANKL et OPG.

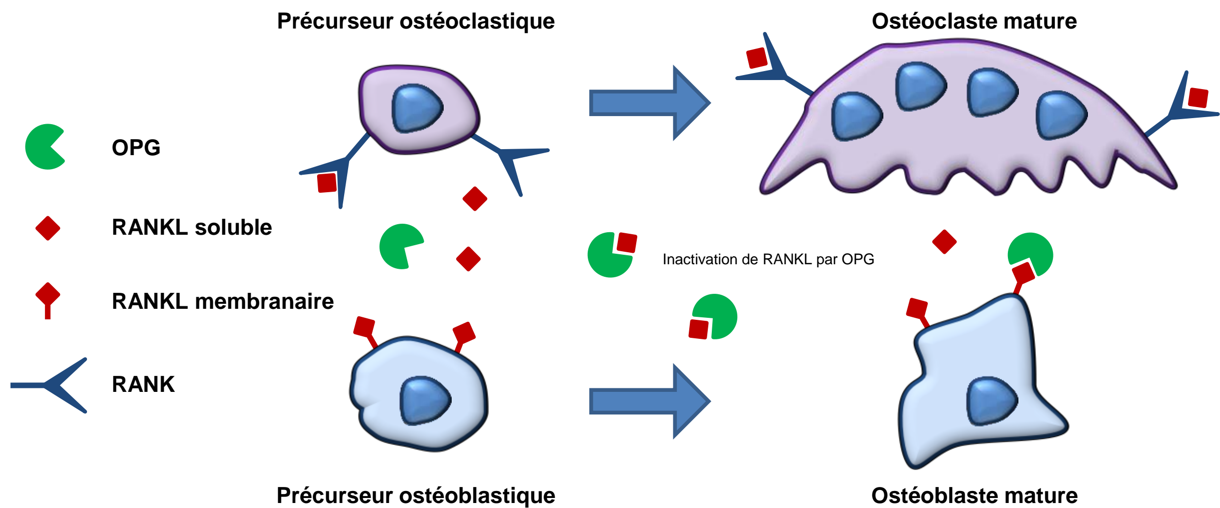

Figure 5. Régulation de l'ostéoclastogénèse par le *receptor activor of NFκB ligand* (RANKL) et l'ostéoprotégérine (OPG). RANKL est sécrété sous deux formes (membranaire et soluble) par les ostéoblastes et les précurseures ostéoblastiques ; il stimule le recrutement et l'activation des ostéoclastes. La forme membranaire de RANKL interagit avec le récepteur mambranaire RANK à la surface des ostéoclastes lors d'un contact direct entre les cellules. La forme soluble (sRANKL) est sécrétée par les ostéoblastes, diffuse dans la matrice extracellulaire et interagit avec les récepteurs RANK des ostéoclastes avoisinants de manière paracrine. L'OPG sécrété également par les ostéoblastes peut fixer RANKL et empêcher son interaction avec RANK. Le ratio entre RANKL et OPG permet ainsi aux ostéoblastes et précurseurs ostéoblastiques de contrôler l'ostéoclastogénèse médiée par RANKL. D'après (Clarke, 2008).

III.B. Infection, réponse inflammatoire et résorption osseuse

L'infection à *Staphylococcus aureus* induit la production de plusieurs cytokines qui jouent un rôle dans la pathogénie des IOA en contribuant de façon directe à la destruction osseuse. Les cytokines de l'inflammation les plus importantes dans la physiologie et la pathologie osseuse sont le *tumour necrosis factor* α (TNFα), l'interleukine (IL) 1β (la forme sécrétée de l'IL1), et l'IL6 (Kwan Tat *et al.*, 2004). Les patients atteints d'infection osseuse présentent une élévation des niveaux plasmatiques de ces trois cytokines (Klosterhalfen *et al.*, 1996; Evans *et al.*, 1998). Des niveaux élevés d'IL1β, d'IL6 et de TNFα sont également retrouvés dans le liquide synovial des patients atteints d'arthrite septique (Saez-Llorens *et al.*, 1990;

Osiri *et al.*, 1998). De plus, certains polymorphismes spécifiques dans les gènes codant l'IL1 et l'IL6 pourraient être associés à un risque accru d'infection osseuse (Tsezou *et al.*, 2008).

III.B.1. Sources de production d'IL1β, d'IL6 et de TNFα au cours des IOA à S. aureus

De façon analogue aux observations réalisées chez les patients infectés, différents modèles animaux d'infection osseuse à *S. aureus* ont montré que cette pathologie induit une augmentation à la fois locale et systémique des concentrations en IL1β, IL6 et TNFα. Des niveaux élevés d'IL1β ont été mesurés dans les tibias de rats inoculés à l'aide de trocards infectés par *S. aureus*, et ces animaux présentaient également une élévation du taux circulant d'IL6 (Garcia-Alvarez *et al.*, 2009). En modèle murin d'ostéomyélite, les niveaux osseux d'IL1β et d'IL6 s'élèvent à la phase précoce de l'infection, alors que le niveau de TNFα augmente plus tardivement (Yoshii *et al.*, 2002). La source de sécrétion locale de ces cytokines n'est pas complètement élucidée. La production d'IL1β a pu être induite ex vivo chez la lignée de cellules ostéoblastiques HOBIT par différents stimuli dont le TNFα (Pivirotto *et al.*, 1995). Cependant, l'infection par *S. aureus* d'ostéoblastes primaires murins, bien qu'elle s'accompagne d'une élévation du niveau transcriptionnel d'IL1, n'est pas associée à la production ni à la sécrétion d'IL1β (Marriott *et al.*, 2002). Chez les cellules ostéoblastiques humaines MG-63, dérivées d'ostéosarcome, ainsi que chez des ostéoblastes différenciés à partir de cellules souches mésenchymateuses, le TNFα n'est détectable qu'à de faibles niveaux (Bu *et al.*, 2003). Dans ce contexte, il est probable que la principale source locale d'IL1β et de TNFα soit l'infiltration du tissu osseux par des cellules immunitaires en réponse à l'infection (Ishimi *et al.*, 1990; Mundy, 1991; Bost *et al.*, 1999; Marriott *et al.*, 2002; Robinson *et al.*, 2007). En revanche, l'IL6 est sécrétée par les ostéoblastes en réponse à de nombreux stimuli dont l'infection à *S. aureus* (Ishimi *et al.*, 1990; Bost *et al.*, 1999).

III.B.2. Impact de la sécrétion de cytokines sur l'ostéoclastogénèse et la résorption osseuse

Ces différentes cytokines jouent un rôle majeur dans le remodelage osseux et la pathogénie de l'infection osseuse. Les modèles de culture cellulaire indiquent que l'IL1 et le TNFα stimulent la prolifération des progéniteurs ostéoclastiques et leur différentiation en ostéoclastes en présence d'ostéoblastes (Pfeilschifter *et al.*, 1989; Mundy, 1991; Tokukoda *et al.*, 2001). L'IL1 et le TNFα stimulent également l'activité de résorption osseuse chez les ostéoclastes ; ce phénomène est observé en l'absence d'ostéoblastes mais semble potentialisé par leur présence (Thomson *et al.*, 1987; Azuma *et al.*, 2000; Taubman et Kawai, 2001). De la même façon, l'IL6 stimule l'activité de résorption osseuse des ostéoclastes en modèle de culture de calvaria murin, et stimule l'ostéoclastogénèse en présence d'ostéoblastes (Ishimi *et al.*, 1990; Kotake *et al.*, 1996). Dans un modèle de périodontite simienne, l'administration locale d'antagonistes de l'IL1 et du TNFα permet de réduire de façon significative l'ostéoclastogénèse et la destruction osseuse (Assuma *et al.*, 1998). Chez la souris, l'administration intra-veineuse d'IL1 et de TNFα augmente la résorption osseuse de façon dose-dépendante (Konig *et al.*, 1988) ; de plus, l'inactivation des récepteurs IL1-R, TNF-R1 et TNF-R2 est associée à une diminution significative du nombre d'ostéoclastes et de la surface de résorption osseuse après injection de lipopolysaccharide (LPS) dans le calvaria de souris (Chiang *et al.*, 1999). Au final, l'IL1, le TNFα et l'IL6 augmentent l'activité ostéoclastique, les deux premiers de façon directe, la dernière de façon indirecte via son action (encore inconnue) sur les ostéoblastes.

III.B.3. Impact de la sécrétion de cytokines sur la différentiation et l'activité des ostéoblastes

L'IL1 et le TNFα inhibent la différentiation des cellules souches mésenchymateuses en ostéoblastes, et abolissent la minéralisation et l'expression des gènes de la phosphatase alcaline et du procollagène I chez ces cellules, bien que seul le TNFα inhibe l'expression des gènes codant l'ostéonectine et

l'ostéopontine (Lacey *et al.*, 2009). Le TNFα abaisse également la production du collagène de type I, de l'ostéocalcine et de la phosphatase alcaline dans différents modèles de culture d'ostéoblastes et d'explants osseux, avec pour conséquence une réduction de la production de matrice osseuse et de sa minéralisation (Canalis, 1987; Smith *et al.*, 1987; Centrella *et al.*, 1988; Nanes *et al.*, 1989; Nanes *et al.*, 1991; Li et Stashenko, 1992; Nanes, 2003).

III.B.4. Déclenchement de la production de cytokines par S. aureus

Plusieurs auteurs ont montré que des extraits de protéines de surface de *S. aureus*, obtenues par extraction saline, stimulent la résorption osseuse et l'ostéoclastogenèse (Nair *et al.*, 1995; Meghji *et al.*, 1998). Cette stimulation est annulée par la neutralisation du TNFα et de l'IL6, alors que la neutralisation de l'IL1 n'a qu'un effet partiel (Meghji *et al.*, 1998). L'effet de ces protéines de surface bactériennes sur l'ostéoclastogénèse et la résorption osseuse n'est pas dépendant de la présence d'ostéoblastes ou de la voie de signalisation RANK (Lau *et al.*, 2006).

L'induction et la sécrétion de ces cytokines en réponse à des molécules bactériennes implique deux grandes classes de récepteurs de reconnaissance de motifs moléculaires (*pattern recognition receptors, PRR*), les Toll-like receptors (TLR) et les NOD-like receptors (NLR). La production de TNFα et d'IL6 par les macrophages murins en réponse à l'infection par *S. aureus* dépend du TLR2, et les souris déficientes en TLR2 présentent une mortalité supérieure aux souris sauvages dans un modèle d'inoculation intraveineuse de *S. aureus* (Hoshino *et al.*, 1999; Takeuchi *et al.*, 1999). L'activation des TLR en réponse à un ligand bactérien a pour effet d'amorcer la production d'IL1β par la cellule en induisant l'expression de la pro-forme inactive de la cytokine (Kahlenberg *et al.*, 2005; Creagh et O'neill, 2006). L'IL1β est synthétisé sous la forme d'un précurseur de 31 kDa, et est clivé par la caspase 1 pour aboutir à une molécule active de 17 kDa. L'activation de la caspase 1, ainsi que les mécanismes en aval qui permettent la maturation et la sécrétion d'IL1β sous forme active, font intervenir un assemblage multi-protéines complexe connu sous le nom d'inflammasome. Ce complexe inclut la caspase 1, la protéine

adaptatrice ASC (*apoptosis-associated speck-like protein containing a caspase recruitment domain*) et une protéine de la classe des NLR, parmi lesquelles quatre membres ont été décrits comme pouvant s'associer aux inflammasomes (Ting *et al.*, 2008). Chaque NLR répond à différents signaux activateurs, et bien que les mécanismes exacts de reconnaissance ne soient pas encore élucidés, différents stimuli ont été identifiés dont la flagelline, la toxine létale de *Bacillus anthracis* ou encore le dipeptide muramyl, un constituant du peptidoglycane des bactéries Gram positif et Gram négatif (Boyden et Dietrich, 2006; Franchi *et al.*, 2006; Miao *et al.*, 2006; Faustin *et al.*, 2007).

S. aureus est un ligand du *NLR family pyrin domain containing 3* (NLRP3, ou cryopyrine). Bien que l'on ait pu établir que le NLRP3 et la protéine ASC sont nécessaires à la sécrétion d'IL1β par les macrophages murins en réponse à *S. aureus* (Mariathasan *et al.*, 2006), le stimuli bactérien est inconnu à ce jour. En particulier, la délétion des gènes bactériens codant les toxines α, β et γ n'a pas d'impact sur la sécrétion de cytokine (Ting *et al.*, 2008). L'inflammasome est impliqué dans le déclenchement de la mort cellulaire en réponse à une infection bactérienne (Marriott *et al.*, 2002); cependant, bien que l'invasion d'ostéoblastes murins par *S. aureus* déclenche leur apoptose, l'implication de l'inflammasome n'a pas été établie (Tucker *et al.*, 2000; Mccall *et al.*, 2008). De plus, la détection de *S. aureus* par le TLR2 aboutit chez les ostéoblastes à une augmentation de transcription d'IL1, sans que soit détectée la forme active IL-1β nécessitant une maturation par l'inflammasome (Marriott *et al.*, 2002). Tout se passe donc comme si l'activation de l'inflammasome en réponse à *S. aureus* est inefficace chez les ostéoblastes ; ainsi, il est peu probable que la détection des *S. aureus* extracellulaires par le TLR2 joue un rôle majeur dans le déclenchement de l'inflammation par les ostéoblastes.

Une réponse cytokinique efficace, avec sécrétion d'IL-6, est en revanche observée chez les ostéoblastes envahis par *S. aureus*. Cette réponse s'accompagne d'une augmentation transcriptionnelle de Nod2, un PRR intracellulaire reconnaissant un motif du peptidoglycane bactérien (Marriott *et al.*, 2005). La sécrétion d'IL-6 après infection par des *S. aureus* capables d'envahir les ostéoblastes est supérieure à celle induite par contact avec des *S. aureus* non invasifs, ce qui confirme que la détection

intracellulaire de *S. aureus* par Nod2 joue un rôle plus important dans l'inflammation que la détection membranaire par le TLR2.

III.B.5. *Voies de signalisation cellulaire associées à l'activation de l'ostéoclastogénèse par les cytokines*

Les voies de signalisation en réponse à l'IL1β et au TNFα aboutissent à l'activation du facteur de transcription NFκB et des signalisations via la *c-Jun N-terminal kinase* (JNK) et la *mitogen-activated protein kinase* p38 (MAPK p38) (Arend *et al.*, 2008; Dinarello, 2009). L'inactivation des sous-unité p50 et p52 de NFκB chez la souris a permis de montrer qu'au moins l'une de ces sous-unités est nécessaire à l'ostéoclastogénèse et à l'activité de résorption osseuse médiée par l'IL1 ; l'activité pro-ostéoclastique de cette cytokine est donc dépendante de NFκB (Xing *et al.*, 2003). Le TNFα peut se lier à deux récepteurs, les TNF-R de type 1 et 2 ; ces récepteurs possèdent des mécanismes de signalisation différents, bien qu'un certain niveau de *crosstalk* existe entre leurs voies de signalisation (Aggarwal, 2003; Wajant *et al.*, 2003). Il est intéressant de noter que la protéine A, un facteur de virulence de *S. aureus* capable de fixer la fraction constante des IgG, est également capable de se lier au TNF-R1 et de déclencher l'inflammation par ce biais (Gomez *et al.*, 2004). Le TNFα potentialise l'activité ostéoclastogénique de RANKL. La production de TNFα par les progéniteurs ostéoclastiques est induite par RANKL, et stimule à son tour la différentiation ostéoclastique à la manière d'une boucle autocrine (Zou *et al.*, 2001).

La voie de signalisation induite par la fixation de l'IL6 à son récepteur IL6-R fait intervenir l'activation des *Janus family tyrosine kinases* (JAK) puis l'activation par phosphorylation des facteurs de transcription de la famille des *signal transducers and activators of transcription* (STAT) (Kwan Tat *et al.*, 2004). L'activation de la résorption osseuse par l'IL6, contrairement à l'IL1β et au TNFα, est indirecte : l'IL6 n'a pas d'effet sur des ostéoclastes isolés, et l'ostéoclastogénèse médiée par l'IL6 nécessite l'expression de l'IL6-R par les ostéoblastes, mais pas par les progéniteurs ostéoclastiques (Hattersley *et al.*, 1988; Udagawa *et al.*, 1995; Kwan Tat *et al.*, 2004).

Au final, il est clair que la triade IL1β, TNFα et IL6 joue un rôle prédominant dans la régulation du remodelage osseux, et que toute perturbation des niveaux d'expression de ces cytokines peut avoir un effet majeur sur ce remodelage. Bien que plusieurs détails mécanistiques soient encore à élucider, il est clairement démontré que l'infection par *S. aureus* déclenche la production locale et systémique de ces trois cytokines via les PRR de l'hôte (Figure 6). L'augmentation de production de ces cytokines conduit alors à un déséquilibre dans l'homéostasie du remodelage osseux en faveur de l'ostéoclastogénèse et de l'activité de résorption osseuse, et aux dépens de la production de matrice osseuse et de sa minéralisation par les ostéoblastes, conduisant *in fine* à la destruction du tissu osseux.

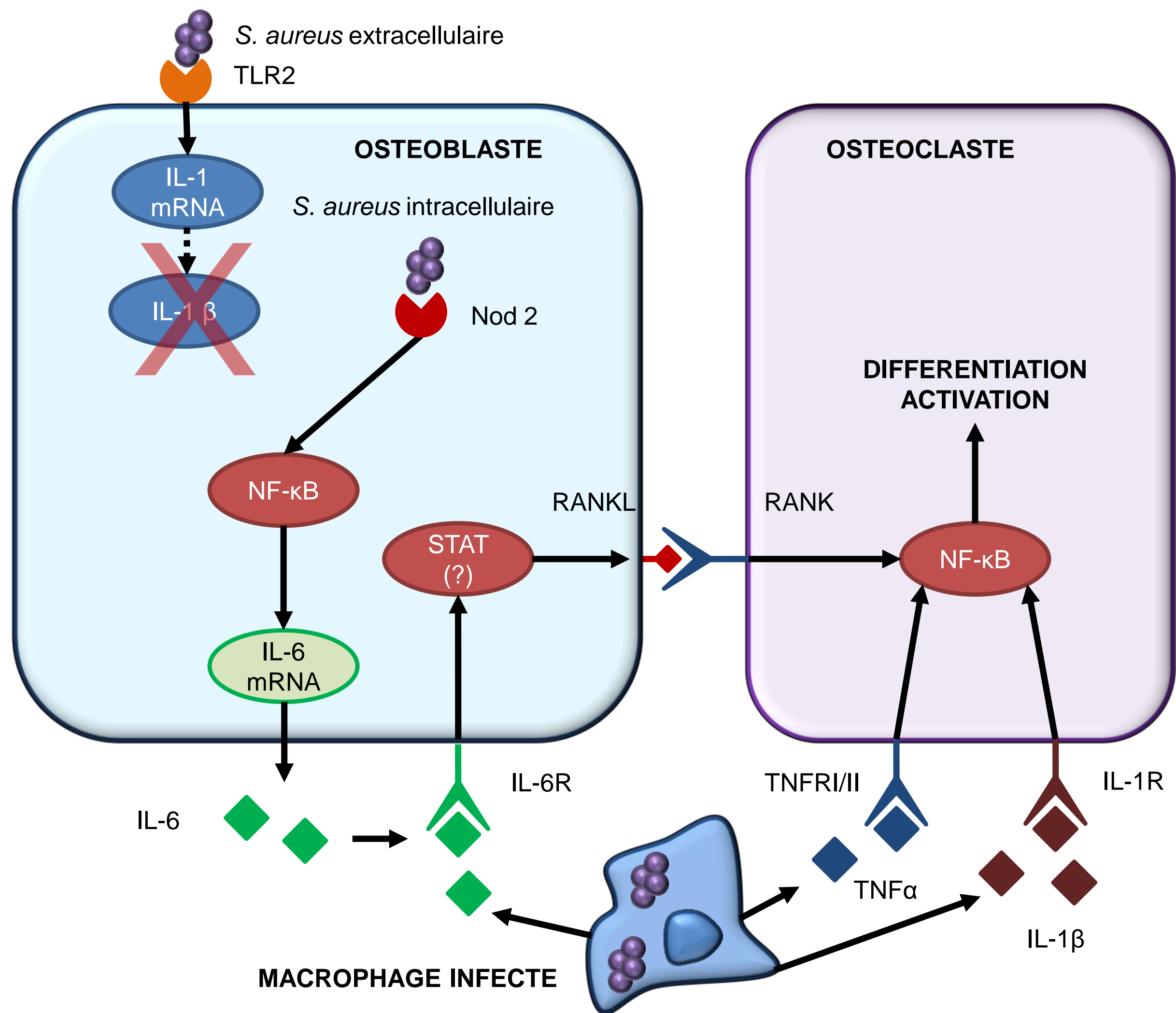

Figure 6. Représentation schématique des voies d'activation de l'ostéoclastogénèse par *S. aureus*. *S. aureus* est détecté par le TLR-2 exprimé à la surface des ostéoblastes. Bien que l'activation du TLR2 s'accompagne d'une augmentation de transcription de l'IL1, la maturation vers la forme active IL-1β, sous contrôle de l'inflammasome, n'est pas détectée chez les ostéoblastes. Les bactéries en position intracellulaire sont détectées par Nod2, dont l'activation conduit via NF-κB à la sécrétion d'IL-6. Cette dernière stimule l'expression de RANKL par les ostéoblastes par une voie non encore élucidée, probablement dépendante de STAT. En aval, l'expression de RANKL active le récepteur RANK exprimé par les ostéoclastes et les précurseurs ostéoclastiques, stimulant ainsi la différentiation et l'activité ostéoclastique via NF-κB. Cette activité est également stimulée par la sécrétion de TNFα et d'IL-1β par les macrophages recrutés par la réponse inflammatoire locale.

III.C. Internalisation de *S. aureus* par les ostéoblastes et persistance intra-cellulaire

Au-delà de sa capacité à induire une réponse inflammatoire qui impacte l'activité des ostéoblastes et des ostéoclastes, *S. aureus* est impliqué dans des interactions de nature plus directe avec les cellules osseuses. L'invasion et la persistance de *S. aureus* chez des cellules de l'hôte qualifiées de « phagocytes non professionnels » est documentée chez de nombreux types cellulaires, notamment les cellules épithéliales, endothéliales et les kératinocytes (Kintarak *et al.*, 2004; Garzoni et Kelley, 2009). Des modèles de culture cellulaire ont permis de démontrer que *S. aureus* envahit les ostéoblastes de différentes espèces comme la souris, la poule et l'homme (Hudson *et al.*, 1995; Ellington *et al.*, 1999; Jevon *et al.*, 1999; Reilly *et al.*, 2000). Par microscopie électronique, Reilly et al. ont pu observer la présence de *S. aureus* à l'intérieur des ostéoblastes et des ostéocytes d'embryons de poulet infectés par *S. aureus*, démontrant ainsi que l'invasion de ces cellules par la bactérie a également lieu in vivo (Reilly *et al.*, 2000).

Certaines données de la littérature suggèrent que l'invasion des ostéoblastes par *S. aureus* a effectivement lieu chez l'homme. En particulier, Bosse *et al.* ont objectivé, à l'intérieur d'ostéoblastes et d'ostéocytes prélevés chez un patient souffrant d'une infection chronique et récurrente de la fibula, la présence de bactéries. Bien qu'aucune identification formelle de *S. aureus* n'ait été réalisée chez ce patient, les bactéries intracellulaires étaient Gram-positif et présentaient une morphologie en cocci évocatrice en microscopie électronique à transmission (Figures 7 et 8) (Bosse *et al.*, 2005). Dans ce contexte, l'hypothèse selon laquelle le caractère intracellulaire facultatif de *S. aureus* fournit à la bactérie une niche de protection, à l'abri du système immunitaire et de la plupart des antibiotiques, a progressivement été acceptée par une majorité d'auteurs ; elle est de plus considérée comme un phénomène explicatif plausible du caractère chronique, récurrent ou indolent de certaines infections osseuses d'une part, et d'autre part du taux élevé d'échec de l'antibiothérapie (Ellington *et al.*, 2003; Ellington *et al.*, 2006). Cependant, la

pertinence clinique de la survie intracellulaire de *S. aureus* dans le cadre des IOA n'a pas encore été démontrée.

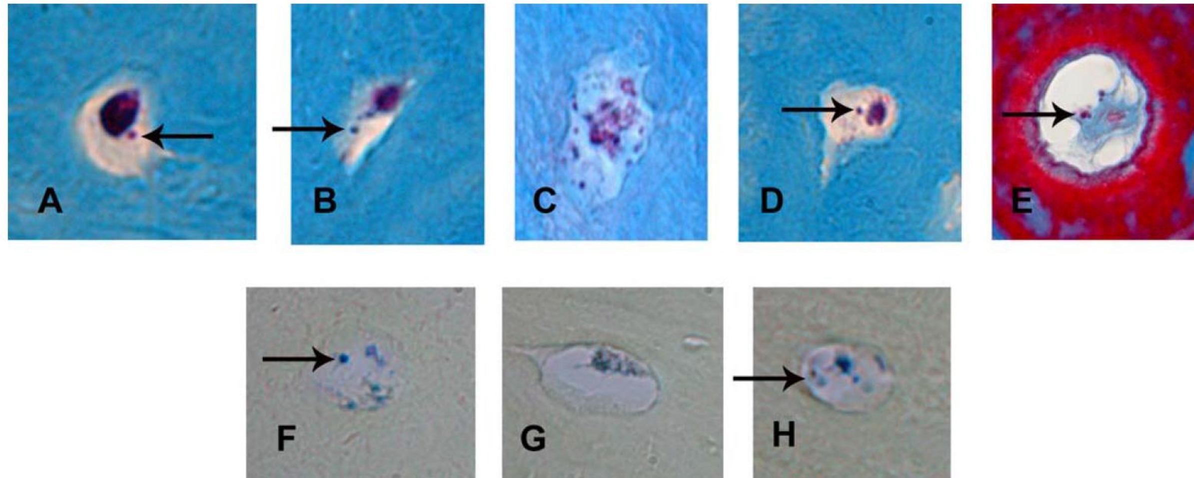

Figure 7. Visualisation en microscopie optique d'ostéocytes contenant des bactéries intracellulaires Gram-positif chez un patient atteint d'infection osseuse chronique. **A** à **E,** coloration au trichrome de Masson. Les ostéocytes sont localisés au sein de vacuoles et sont entièrement entourés de tissu osseux. Les bactéries sont visualisées sous la forme de points (flèches) localisés dans le cytoplasme. **F** à **H**, coloration de Gram. Les bactéries intracellulaires sont Gram-positives. Grossissement x1000. D'après (Bosse *et al.*, 2005).

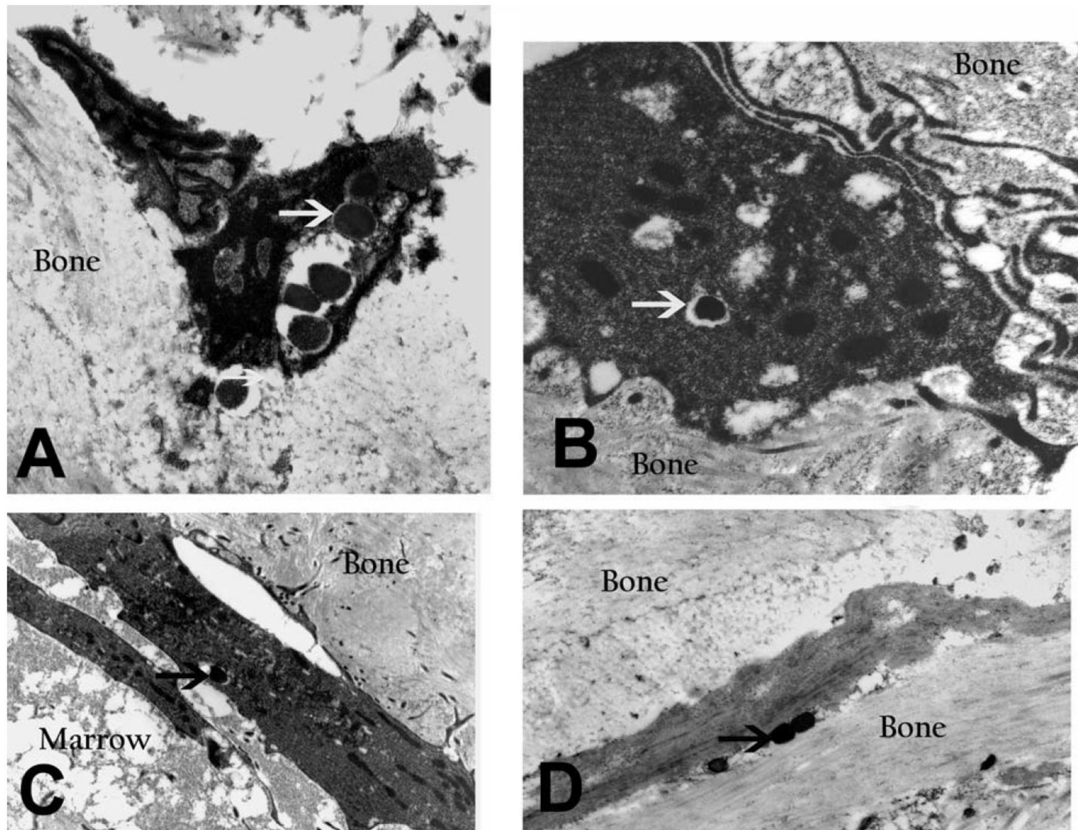

Figure 8. Visualisation en microscopie électronique à transmission des cocci en position intracellulaires au sein d'ostéoblastes et d'ostéocytes, ou libres dans la matrice osseuse. **A** et **B**, ostéocytes enclavés dans la matrice osseuse et contenant des cocci au sein de vacuoles intracytoplasmiques (flèches). **C**, ostéoblastes contenant un coque intracellulaire. **D**, bactéries libres au sein de la matrice osseuse. Grossissement x15000 (A, B, D) ou x5000 (C). D'après (Bosse *et al.*, 2005).

III.C.1. L'invasion cellulaire par S. aureus *est médiée par les FnBP, la fibronectine et les intégrines α5β1*

L'internalisation de *S. aureus* par différents types cellulaires, dont les ostéoblastes, nécessite l'expression à la surface bactérienne de protéines liant la fibronectine (FnBP) (Sinha *et al.*, 1999; Ahmed *et al.*, 2001; Garzoni et Kelley, 2009). Les FnBP appartiennent à une classe d'adhésines désignées sous le terme de *microbial surface components recognizing adhesive matrix molecules* (MSCRAMM), capables de lier un large panel de protéines de la matrice extracellulaire, notamment la fibronectine, le fibrinogène, le collagène, l'élastine ou la sialoprotéine osseuse (Hauck et Ohlsen, 2006). Des mutants de *S. aureus* dont les gènes codant les deux

FnBP, *fnb*A et *fnb*B, ont été inactivés, présentent une capacité d'invasion des cellules eucaryotes fortement atténuée (Sinha *et al.*, 1999; Ahmed *et al.*, 2001). L'invasion fait également intervenir la fibronectine et l'intégrine α5β1 de l'hôte (Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999; Fowler *et al.*, 2000). *S. aureus* fixe la fibronectine via les FnBPs exprimés à sa surface ; la fibronectine joue alors un rôle de pont entre les FnBPs et l'intégrine α5β1, cette dernière agissant comme un déclencheur de la phagocytose (Sinha *et al.*, 1999). Cependant, bien que la présence de FnBP soit nécessaire à une internalisation efficace, cette molécule ne rend pas compte à elle seule de l'importante variabilité des taux d'internalisation observés selon les souches de *S. aureus* (Ahmed *et al.*, 2001). Des mécanismes d'invasion indépendants de la fibronectine semblent exister ; en effet, un mutant Δ*fnb*A/B de *S. aureus* est toujours capable d'envahir des kératinocytes, et cette invasion n'est pas abolie par l'inactivation de la liaison fibronectine - α5β1 (Kintarak *et al.*, 2004). Le niveau d'expression du facteur de transcription alternatif σ^B impacte le niveau d'expression de *fnb*A et la capacité des souches de *S. aureus* à lier la fibronectine ; enfin, l'expression de σ^B est corrélée au niveau d'internalisation de *S. aureus* par les ostéoblastes, ce qui suggère que la surexpression des FnBP médiée par σ^B facilite l'invasion (Mitchell *et al.*, 2008).

III.C.1.a. Structure des FnBP et nature de leur liaison à la fibronectine

Les protéines de surface FnBPA et B contiennent chacune un site de liaison à la fibronectine qui se compose d'une succession de motifs répétés, partiellement variables, d'environ 40 AA (Massey *et al.*, 2001). Chez FnBPA, chacun des 11 motifs peptidiques présente une affinité variable vis-à-vis de la fibronectine. L'étude de la capacité de liaison à la fibronectine de mutants de *S. aureus* présentant une inactivation de motifs spécifiques sur FnBPA a permis de déterminer que les motifs 1, 4, 5, et 9-11 présentaient une affinité élevée (leur délétion impacte fortement la fonction de FnBPA), alors que les motifs 2, 3, et 6-8 avaient une faible affinité (leur délétion impacte peu la fonction de FnBPA) (Massey *et al.*, 2001; Edwards *et al.*, 2010). Les mêmes auteurs ont montré que la présence d'au moins un motif à forte affinité était nécessaire à l'invasion de cellules endothéliales par *S. aureus*, et que cette invasion était facilitée par la présence de multiples motifs répétés. La

pertinence in vivo de ces observations a été démontrée en modèle de sepsis murin (Edwards *et al.*, 2010). Ainsi, la capacité d'invasion de *S. aureus* est corrélée au nombre de motifs répétés portés par FnBPA.

En réalité, la multiplication des motifs répétés au site actif de FnBPA n'a pas pour fonction d'augmenter l'affinité de la liaison à la fibronectine, mais de multiplier le nombre de molécules de fibronectine liées par une même molécule de FnBPA. L'étude par cristallographie des liaisons FnBPA-fibronectine a permis de montrer que la majorité des motifs répétés était capable de lier une molécule de fibronectine (Bingham *et al.*, 2008). Ainsi, une FnBPA sauvage comprenant 11 motifs est capable de lier 9 molécules de fibronectine. L'expression des FnBPA à la surface de *S. aureus* induit donc le recrutement de fibronectine avec une très forte densité. A la surface la cellule hôte, cette forte densité de fibronectine se traduit par un recrutement focal et dense des intégrines α5β1 reconnaissant chacune une molécule de fibronectine. Avant de poursuivre sur les mécanismes moléculaires déclenchés en aval par l'activation des intégrines α5β1, il est nécessaire d'esquisser un modèle explicatif fonctionnel de cette activation au niveau cellulaire. La fibronectine par elle-même ne déclenche pas l'activation de l'endocytose par la cellule-hôte. Le détournement de la liaison fibronectine - α5β1 par la bactérie repose non pas sur la reconnaissance par la cellule hôte de molécules de fibronectine prises individuellement, mais sur la présentation simultanée d'une grande quantité de molécules de fibronectine regroupées sur une surface très réduite (soit la surface d'une bactérie ou d'un cluster de bactéries). Sur le plan fonctionnel, le pontage fibronectine - α5β1 est impliqué dans l'adhésion entre cellules eucaryotes, et ce pontage implique une force de traction centripète du cytosquelette (cortex d'actine) exercée sous la surface cellulaire et nécessaire à la cohésion et à l'adhésion intercellulaire (Fassler et Meyer, 1995; Berger *et al.*, 2003). Cependant, lorsque la fibronectine n'est pas présentée de façon diffuse à la surface d'une cellule eucaryote, mais de façon très concentrée à la surface d'une bactérie, les forces de traction centripètes sont également concentrées sur une surface réduite. En conséquence apparaissent des protrusions membranaires venant recouvrir la surface bactérienne, ainsi qu'une invagination attirant la bactérie vers le cytoplasme cellulaire. La poursuite de ce phénomène aboutit à la séquestration de la bactérie dans une vacuole de membrane plasmique, désignée sous le terme de phagosome par

analogie avec le phénomène de phagocytose observé par chez les phagocytes professionnels (Figure 9).

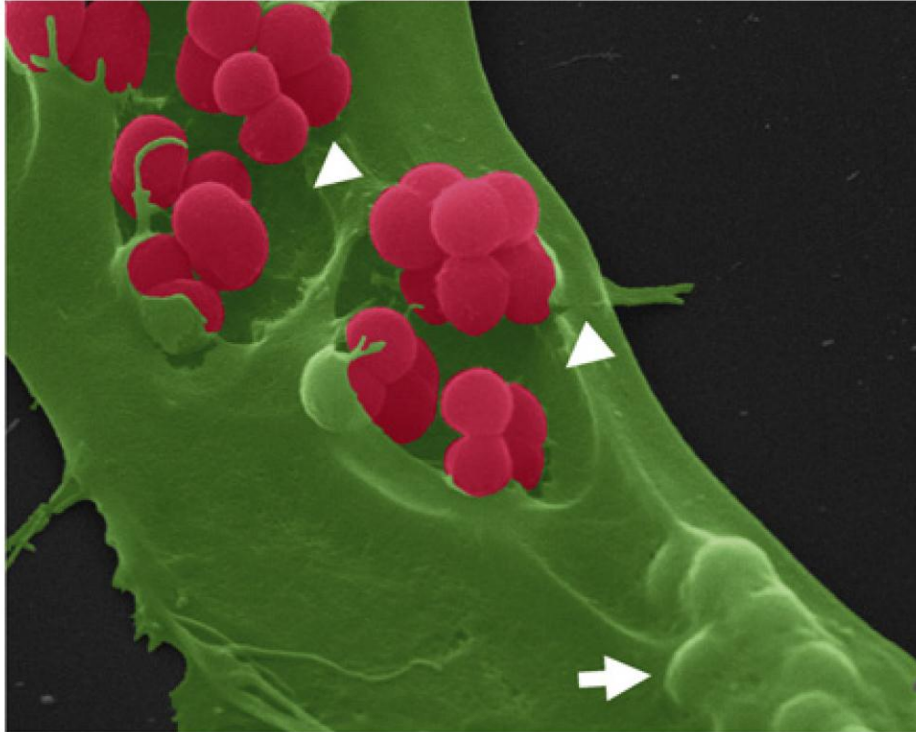

Figure 9. L'invasion cellulaire par *S. aureus* met en jeu des invaginations et protrusions membranaires. Visualisation par microscopie électronique à balayage avec pseudocouleurs (vert, surface cellulaire ; rouge, *S. aureus*) de l'invasion de fibroblastes murins par *S. aureus*. Après 1h de coculture de fibroblastes avec *S. aureus* à une MOI de 20, certaines bactéries sont déjà internalisées et sont visibles sous la membrane plasmique (flèche). Les autres bactéries sont en cours d'internalisation via de larges zones d'invagination formées sous la zone d'adhésion bactérienne (têtes de flèche). D'après (Hoffmann *et al.*, 2011).

III.C.1.b. Déclenchement du remodelage de l'actine par la liaison *S. aureus* – fibronectine – intégrine α5β1

L'internalisation de *S. aureus* médiée par l'intégrine α5β1 est associée à un remodelage de l'actine du cytosquelette (Figure 10) (Agerer *et al.*, 2005). La protéine *integrin-linked kinase* (ILK) joue un rôle de premier intermédiaire entre l'α5β1 et le cytosquelette. L'ILK interagit avec le domaine cytoplasmique des intégrines β et est activée par la liaison de l'α5β1 à la fibronectine. Cette protéine ILK est nécessaire à l'internalisation de *S. aureus* par les cellules épithéliales (Wang *et al.*, 2006). Son

activation déclenche le recrutement de la *focal adhesion kinase* (FAK, ou PTK2) (Boulter et Van Obberghen-Schilling, 2006). Le recrutement de protéines d'adhésion focale dont la tensine, la zyxine et la vinculine est également déclenché au site d'adhésion bactérienne. FAK est alors activé par phosphorylation ; son action, combinée à celle des tyrosine kinases de la famille Src, semble essentielle car des cellules inactivées pour FAK internalisent *S. aureus* de façon peu efficace (Figure 11). La suite du phénomène d'invasion fait intervenir la phosphorylation de substrats de FAK dont la cortactine, dont le blocage réduit également le niveau d'internalisation. On en conclut que les voies de signalisation de l'intégrine α5β1, qui font intervenir l'ILK, FAK et la cortactine, jouent un rôle important dans l'invasion des cellules eucaryotes par *S. aureus*, tout au moins chez certains types cellulaires (Agerer *et al.*, 2005; Wang *et al.*, 2006).

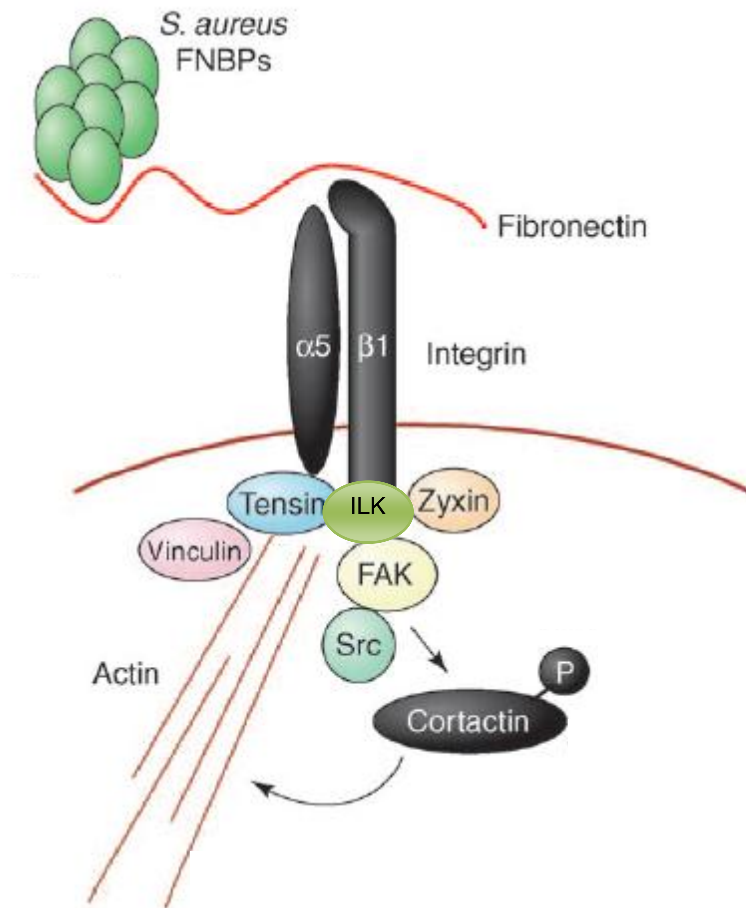

Figure 10. Voies de signalisation cellulaire déclenchées par le pontage *S. aureus* – fibronectine – intégrine α5β1. *S. aureus* se fixe à la fibronectine par ses protéines de surface FnBP. La fibronectine associée à la surface bactérienne est reconnue par son récepteur à la surface de la cellule hôte, l'intégrine α5β1. La liaison de multiples molécules de fibronectine induit le regroupement (clustering) des intégrines et le recrutement local, via ILK, de protéines de structure dont la tensine, la vinculine et la zyxine, ainsi que des enzymes de signalisation FAK et les tyrosine kinases de la famille Src. L'activité combinée de FAK et Src aboutit à la phosphorylation de différents effecteurs d'aval dont la cortactine. L'impact de cette dernière sur l'invasion bactérienne est très probablement lié aux réarrangements du cytosquelette d'actine. D'après (Hauck et Ohlsen, 2006).

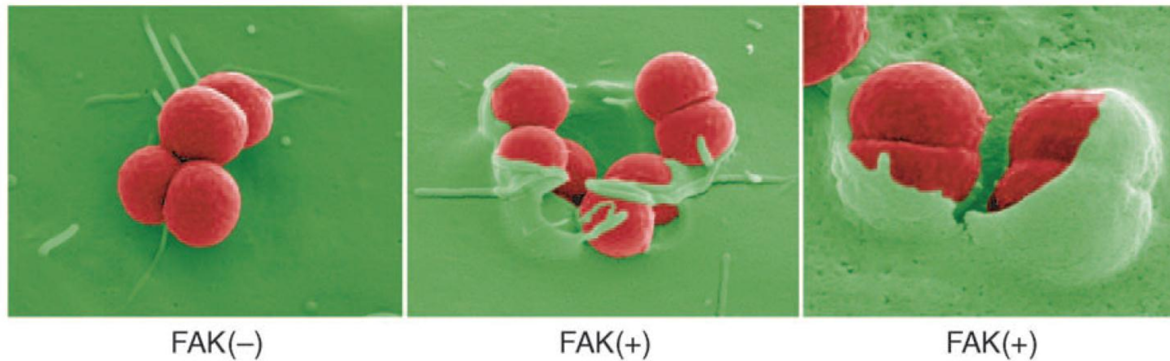

Figure 11. Les cellules n'exprimant pas la *focal adhesion kinase* (FAK) n'internalisent pas *S. aureus*. Des cellules déficientes en FAC [FAC(-)] ou recomplémentées en FAC [FAC(+)] infectées par *S. aureus* ont été analysées par microscopie électronique à balayage (grossissement x20 000). Les pseudocouleurs représentent la surface cellulaire en vert et les bactéries en rouge. Bien que *S. aureus* soit capable d'adhérer aux cellules FAC(-), les protrusions et l'invagination membranaires initiant la phagocytose ne sont observées que chez les cellules FAK(+). D'après (Hauck et Ohlsen, 2006).

III.D. Induction de la mort cellulaire par *S. aureus* et échappement phagosomal

Bien que peu de données soient disponibles sur l'induction de la mort chez les ostéoblastes infectés par *S. aureus*, cette question a été étudiée chez d'autres types cellulaires. Les données de la littérature suggèrent que la survenue de la mort chez les cellules infectées par *S. aureus* est dépendante du type de cellule hôte, de la souche infectante et de l'inoculum bactérien utilisé (*multiplicity of infection*, MOI). Il en va de même pour la voie de mort, apoptotique ou nécrotique, les mécanismes moléculaires et surtout les facteurs de virulence de *S. aureus* impliqués dans la mort cellulaire.

Les expériences menées sur les cellules endothéliales ont montré que les souches présentant à la fois un phénotype invasif et hémolytique (dont la souche 6850 et les souches de *sequence type* (ST) 239) déclenchent l'apoptose des cellules infectées, contrairement aux souches hémolytiques et non-invasives, ou invasives et non-hémolytiques (Haslinger-Loffler *et al.*, 2005). L'induction de la mort cellulaire

chez les cellules endothéliales infectées s'accompagne d'une activation des caspases ; cependant, l'inactivation des caspases ne permet pas d'inhiber la mort cellulaire chez ces cellules (Haslinger-Loffler *et al.*, 2005), contrairement à ce qui est observé chez les monocytes ou les lymphocytes T de la lignée Jurkat (Haslinger *et al.*, 2003). Enfin, l'activité caspase est indétectable chez les cellules mésothéliales humaines tuées par *S. aureus*, ce qui suggère l'implication de voies de mort complètement indépendantes des caspases (Haslinger-Loffler *et al.*, 2006).

III.D.1. Induction de mort chez les cellules eucaryotes par contact direct avec S. aureus et via l'action de toxines bactériennes

Le contact direct entre *S. aureus* et les ostéoblastes déclenche l'expression par ces derniers du *tumour necrosis factor apoptosis inducing ligand* (TRAIL) (Alexander *et al.*, 2003). TRAIL fait partie des cytokines de la famille du TNF et se lie à deux récepteurs à domaine de mort, les TRAIL-receptors de type 1 et 2. L'activation de ces récepteurs déclenche le recrutement de la protéine adaptatrice *Fas-associated protein with death domain* (FADD) qui active à son tour les caspases 8 et 10. Cette activation déclenche l'engagement de la cellule sur la voie apoptotique (Mahalingam *et al.*, 2009). Le facteur TRAIL sécrété par des ostéoblastes infectés par *S. aureus* déclenche ainsi l'apoptose des ostéoblastes non infectés par la voie de la caspase 8 (Alexander *et al.*, 2003). Ces derniers, lorsqu'ils sont cultivés en présence d'ostéoblastes infectés, expriment également TRAIL (Reott *et al.*, 2008). Ces observations suggèrent que l'apoptose des ostéoblastes infectés par *S. aureus*, ainsi que celle des cellules avoisinantes et non infectées, participent à la perte de tissu osseux au cours de l'ostéomyélite (Henderson et Nair, 2003).

L'induction de la mort cellulaire par *S. aureus* semble dépendante de l'expression du régulateur staphylococcique majeur *accessory gene regulator* (*agr*), qui module schématiquement la balance d'expression entre les exotoxines bactériennes et les protéines de surface. En effet, les mutants Δ*agr* de *S. aureus* sont incapables de déclencher la mort des cellules infectées (Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008). A l'inverse, des mutants inactivés pour *sar*A, un autre

régulateur de virulence de *S. aureus*, sont toujours capables de déclencher l'apoptose (Haslinger-Loffler *et al.*, 2005; Jarry *et al.*, 2008).

Chez les monocytes et polynucléaires neutrophiles humains, deux exotoxines staphylococciques déclenchent à elles seules la mort cellulaire : l'α-toxine et la leucocidine de Panton-Valentine (Bantel *et al.*, 2001; Haslinger *et al.*, 2003; Genestier *et al.*, 2005). Des concentrations élevées d'α-toxine induisent majoritairement la nécrose cellulaire, alors que des concentrations plus faibles induisent l'apoptose (Haslinger *et al.*, 2003). L'α-toxine mise en présence de monocouches cellulaires de kératinocytes est capable de former des pores membranaires perméables aux ions monovalents, aboutissant à la mort des cellules (Walev *et al.*, 1993). Cependant, ces études ont employé des surnageants de culture ou de l'α-toxine purifiée comme source de toxine, et leurs résultats ne sont pas obligatoirement extrapolables à la mort cellulaire induite spécifiquement par les *S. aureus* intracellulaires.

III.D.2. Stratégies de survie des S. aureus intracellulaires : résistance à l'oxydation, détournement de l'autophagie et échappement pagosomal

L'endocytose déclenchée par *S. aureus* chez les phagocytes non professionnels est analogue à plusieurs titres au phénomène de phagocytose des cellules immunes. Chez les cellules immunes comme chez les cellules non-immunes comme les ostéoblastes, la vacuole d'endocytose, ou phagosome, est destinée à fusionner avec un lysosome pour devenir un phagolysosome. La survie de *S. aureus* dépend donc de sa capacité à résister à ces mécanismes de défense, soit en assurant sa survie à l'intérieur même du phagosome, soit en détruisant ce phagosome pour permettre son relargage à l'état libre dans le cytoplasme de la cellule hôte. La survie intra-phagosomale de *S. aureus* repose sur deux mécanismes identifiés à ce jour, la résistance aux radicaux libres et le détournement des mécanismes d'autophagie.

III.D.2.a. Résistance à la fusion phagosome-lysosome

Lors de la fusion phagosome-lysosome, les premiers changements environnementaux auxquels *S. aureus* est confronté sont la diminution du pH et l'augmentation du potentiel oxydant apportée par les radicaux libres issus d'H2O2. Un des régulateurs de *S. aureus* impliqués dans les voies de réponse au stress, *rsb*U (Novick, 2003), a pour rôle de stimuler la production de staphyloxanthine, un anti-oxydant qui facilite la survie bactérienne en neutralisant l'H2O2 (Olivier *et al.*, 2009).

III.D.2.b. Détournement des mécanismes d'autophagie

L'autophagie est un processus permettant de réguler la biomasse intracellulaire par une voie de contrôle qualitative et quantitative. Ce processus peut également jouer un rôle de défense contre les pathogènes intracellulaires ; l'autophagie dans ce contexte prend le nom de xénophagie (Deretic, 2011). Schématiquement, l'autophagie est initiée par le recrutement d'une vésicule issue du réticulum endoplasmique et désignée pré-autophagosome. Après reconnaissance, directe ou indirecte, de la bactérie par le pré-autophagosome, la membrane vésiculaire subit une élongation qui circonscrit la bactérie ou le phagosome la contenant. Cette élongation est suivie d'un cloisonnement qui aboutit à la formation de l'autophagosome proprement dit. Une étape de maturation, qui aboutit à la lyse de la bactérie, fait intervenir la fusion de l'autophagosome avec un lysosome. Cependant, une particularité de l'interaction autophagosome-*S. aureus* est l'incapacité du processus d'autophagie à tuer la bactérie. *S. aureus* n'est pas lysé par l'autophagosome et peut même se multiplier à l'intérieur de façon plus efficace qu'en l'absence d'autophagie. Il semble donc dans ce cas que l'autophagie, loin de remplir son rôle xénophage, fournit au contraire à *S. aureus* une niche de réplication à l'abri des autres mécanismes de défense de la cellule hôte (Deretic, 2011).

La démonstration de ce phénomène a été apportée par des expériences croisées de gain et perte de fonction. Dans un modèle d'infection de cellules HeLa par des *S. aureus* sauvages et leurs mutants respectifs Δ*agr*, Schnaith et al. ont observé que les mutants Δ*agr* ne déclenchaient pas l'autophagie et présentaient une survie intracellulaire diminuée (Schnaith *et al.*, 2007). L'addition de rapamycine, un

inducteur d'autophagie, restaurait la survie intracellulaire des mutants Δ*agr*. Inversement, l'addition de wortmannine, un inhibiteur de l'autophagie, diminuait la survie des souches sauvages. Enfin, la multiplication intra-autophagosomale de *S. aureus* aboutissait à la rupture de l'autophagosome, à la libération des bactéries à l'état libre dans le cytoplasme de la cellule hôte, et enfin à la mort cellulaire par une voie indépendante des caspases, assimilée à de la nécrose. *S. aureus*, à la condition de posséder un régulateur *agr* fonctionnel, semble donc capable de détourner à son profit le phénomène d'autophagie pour se répliquer et aboutir à la mort de la cellule hôte (Schnaith *et al.*, 2007).

III.D.2.c. Echappement phagosomal

La destruction du compartiment phagosomal a été décrite chez plusieurs pathogènes dont *Listeria monocytogenes*, *Shigella* et *Rickettsia* (Hybiske et Stephens, 2008). Cette destruction repose sur l'expression de toxines formant des pores (*pore-forming toxin*, PFT) et de phospholipases dégradant les membranes lipidiques. Il a été proposé dès 1998 que *S. aureus* était capable de s'échapper des phagosomes chez les phagocytes non professionnels (Bayles *et al.*, 1998). L'échappement phagosomal semble être un pré-requis à l'induction de la mort cellulaire ; cette assertion repose sur le fait que l'expression de lysostaphine (une glycyl-glycyl endopeptidase bactéricide pour *S. aureus*) dans le cytoplasme de cellules HeLa protège ces dernières de l'induction de la mort par les *S. aureus* ATCC29213 internalisés (Klein *et al.*, 2006). L'échappement phagosomal a initialement été attribué à l'expression d'α-toxine car seules les souches invasives et hémolytiques induisent l'apoptose des cellules endothéliales (Haslinger-Loffler *et al.*, 2005). Cependant, des travaux plus récents ont montré que d'autres toxines dont la β-toxine et la δ-toxine pourraient être impliquées (Giese *et al.*, 2009; Giese *et al.*, 2011).

L'α-toxine, codée par le gène *hla*, est une PFT qui possède des propriétés hémolytiques et cytotoxiques. Le gène *hla* est présent chez virtuellement toutes les souches de *S. aureus*, cependant son niveau d'expression est variable et sous le contrôle des régulateurs *agr*, *sar*A et *sae*RS (Haslinger-Loffler *et al.*, 2005;

Montgomery *et al.*, 2008; Thoendel *et al.*, 2011). L'α-toxine est sécrétée sous forme de monomères qui s'associent en homoheptamère à la surface des cellules-cibles, formant ainsi un pré-pore, puis un pore transmembranaire en tonneau beta (Bhakdi et Tranum-Jensen, 1991). Ce pore transmembranaire crée un canal perméable à l'eau de 14 Å de diamètre permettant le passage de molécules jusqu'à 2 kD (Menestrina, 1986; Song *et al.*, 1996). L'implication de l'α-toxine dans la destruction phagosomale et la mort des cellules infectées par *S. aureus* a été initialement démontrée chez des cellules déficientes en *cystic fibrosis transmembrane conductance regulator* (CFTR) (Jarry et Cheung, 2006; Jarry *et al.*, 2008). Cependant, ce rôle de l'α-toxine était spécifique des cellules CFT1 (CFTR-déficientes) et n'était pas reproduit chez des cellules sauvages ou recomplémentées en CFTR (Jarry et Cheung, 2006; Giese *et al.*, 2009; Giese *et al.*, 2011). Ces résultats suggèrent un rôle de l'α-toxine dans la persistance de *S. aureus* chez les patients atteints de mucoviscidose, et montrent également que l'α-toxine n'est probablement pas la seule responsable de l'échappement phagosomal et de la mort cellulaire chez les cellules non déficientes en CFTR.

La β-toxine est une exotoxine de *S. aureus* possédant une activité sphingomyélinase (Huseby *et al.*, 2007). Le gène *hlb* est présent chez toutes les souches de *S. aureus* ; cependant, chez la majorité des souches d'origine humaine, ce gène est inactivé par l'insertion dans le cadre de lecture d'un bactériophage de la classe des *hlb-converting phages* (Carroll *et al.*, 1993). Par ailleurs, la sphingomyéline est un constituant membranaire des cellules eucaryotes ; son hydrolyse produit un second messager, la céramide, impliqué dans le déclenchement de l'apoptose, et il a été proposé que l'activité sphingomyélinase de la β-toxine intervienne par ce biais dans la mort cellulaire induite par *S. aureus* (Bayles *et al.*, 1998).

La δ-toxine est un petit peptide amphipatique dont le gène *hld* est porté par un des effecteurs du régulateur *agr*, l'ARNIII. Elle possède des propriétés lytiques vis-à-vis des protoplasmes, des mitochondries ainsi que des lysosomes. Ces propriétés, ainsi que la dépendance de l'échappement phagosomal vis-à-vis d'*agr*, ont permis de suggérer un rôle de la δ-toxine dans la destruction de la membrane phagosomale. Ce rôle a été récemment démontré par Giese et al. (Giese *et al.*, 2011). Le taux

d'échappement phagosomal chez la souche *S. aureus* RN4220 était significativement plus élevé que chez son mutant isogénique Δ*hld*. De plus, l'implication de la δ-toxine dans l'échappement phagosomal n'était observé que chez les souches produisant une β-toxine fonctionnelle, ce qui indique qu'une synergie entre β- et δ-toxine est nécessaire à la destruction du phagosome. Cependant, l'échappement phagosomal induit par le couple β-toxine / δ-toxine n'était associé à la mort de la cellule hôte que chez certaines des souches étudiées, ce qui suggère que d'autres facteurs bactériens non encore décrits sont également nécessaires pour déclencher la mort, même en présence d'un échappement phagosomal efficace (Giese *et al.*, 2011).

III.E. Régulation de l'expression des déterminants de virulence chez *S. aureus*

La virulence chez *S. aureus* repose sur l'expression de protéines membranaires et d'exotoxines protéiques dont certains représentants majeurs ont été décrits au cours des chapitres précédents. Les niveaux d'expression de ces déterminants de virulence sont contrôlés par un réseau complexe et interconnecté de régulateurs (Thoendel *et al.*, 2011). Ces régulateurs permettent à la bactérie d'adapter sa virulence en fonction des conditions environnementales et de sa phase de croissance. Bien qu'il existe plus de vingt régulateurs décrits chez *S. aureus*, nous ne présenterons ici que les régulateurs d'importance majeure pour l'expression des exotoxines et des FnBP : *agr*, *sar*A et *sae*RS.

III.E.1. L'accessory gene regulator (agr)

Le système *agr* est le principal régulateur de *S. aureus* et le plus étudié à ce jour. Ce système de quorum sensing à deux composants (senseur membranaire et

effecteur) code son propre facteur d'activation, le peptide auto-inducteur. Lors de la phase exponentielle de croissance, la faible densité bactérienne s'accompagne d'une faible concentration en peptide auto-inducteur, avec pour conséquence un bas niveau d'activité *agr*. Lorsque la densité bactérienne augmente (phase post-exponentielle), l'augmentation concomitante de la concentration en peptide inducteur aboutit à l'activation d'*agr* (Thoendel *et al.*, 2011). Cette activation marque ainsi le passage de la phase de croissance exponentielle (colonisation) à la phase post-exponentielle ou stationnaire, dans laquelle les ressources disponibles viendront à manquer. Le système *agr* réprime la transcription de différentes protéines membranaires, notamment les FnBP et la protéine A, et active l'expression d'exotoxines comme l'α-toxine et la β-toxine. Une conséquence de cette dualité fonctionnelle est que l'expression des adhésines de surface de *S. aureus*, nécessaires à la colonisation tissulaire, est supérieure durant la phase initiale de colonisation ; lorsque la densité bactérienne augmente, l'activation d'*agr* stimule l'expression des exotoxines associées à la destruction tissulaire et à la libération de nouvelles ressources pour la bactérie.

Le locus *agr* comprend cinq gènes, *agr*A, *agr*B, *agr*C, *agr*D, et *hld*, portés par deux produits de transcription, les ARNII et III, chacun sous le contrôle de leur propre promoteur, respectivement P2 et P3 (Peng *et al.*, 1988). L'ARNII porte les cadres de lecture des protéines AgrA à D. AgrD est le précurseur du peptide auto-inducteur, excrété par le transporteur membranaire AgrB ; AgrC est un senseur membranaire activé par le peptide auto-inducteur. AgrC active AgrA par phosphorylation ; ce dernier active alors le promoteur P3 et la transcription de l'ARNIII. Cet ARN régulateur est l'effecteur final du système *agr* ; bien que majoritairement non traduit, il porte cependant un court cadre de lecture codant la δ-toxine, laquelle n'est pas impliquée dans la régulation (Janzon *et al.*, 1989). Bien que la majorité des effets transcriptionnels et post-transcriptionnels du système *agr* soient imputables à l'action de l'ARNIII, la protéine AgrA possède également une cible en dehors du promoteur P2 : elle active la transcription d'exotoxines protéiques analogues à la δ-toxine, les *phenol-soluble modulins* (Thoendel *et al.*, 2011).

Une méthode de typage de *S. aureus* est basée sur l'amplification de la séquence du locus *agr*. En effet, un polymorphisme dans la séquence protéique du

peptide auto-inducteur permet de définir quatre groupes *agr*. Le peptide auto-inducteur d'un groupe *agr* donné active la réponse *agr* des souches du même groupe et inhibe cette réponse chez les souches des autres groupes. La divergence des groupes *agr* est un événement évolutif ancien qui permet de diviser *S. aureus* en quatre fonds génétiques distincts, apparemment en compétition les uns avec les autres (Jarraud *et al.*, 2002).

III.E.1. ***Le régulateur* sar*A***

La protéine *staphylococcal accessory regulator* A (SarA) est encodée par trois produits de transcription différents situés sur l'opéron *sar*A et possédant chacun leur propre promoteur (Bayer *et al.*, 1996). Chaque promoteur est activé par un facteur σ différent, dont σ^B, ce qui permet à SarA d'être exprimée à toutes les phases de croissance. Ce régulateur active de nombreux promoteurs en se liant à des séquence d'ADN spécifiques, désignées sous le terme de *Sar boxes*. Des *Sar boxes* sont présentent sur les promoteurs P2 et P3 d'*agr* (Cheung *et al.*, 1997; Chien *et al.*, 1998), ainsi que sur les promoteurs de la protéine A, des FnBP, de l'α-toxine et de la β-toxine (Chien *et al.*, 1999).

III.E.2. ***Le régulateur* sae*RS***

Le locus *S. aureus exoprotein expression* (*sae*RS) code un système à deux composants, SaeR (régulateur) et SaeS (senseur), régulant positivement la transcription de l'α-toxine, de la β-toxine, de la coagulase, de la protéine A et de la DNAse. Cette régulation est de nature directe et ne fait pas intervenir de modification du niveau d'expression d'*agr* ou du régulateur *sar*A (Giraudo *et al.*, 1997; Giraudo *et al.*, 1999). La nature du ligand de SaeS reste inconnue à ce jour (Sun *et al.*, 2010).

III.F. L'adaptation bactérienne à l'infection chronique : les *small colony variants*

La première description d'une souche de *Staphylococcus aureus* présentant un phénotype variant avec présence de microcolonies à croissance ralentie *in vitro* remonte à plus d'un siècle. Il aura cependant fallu attendre les années 2000 pour que les efforts de recherche se concentrent à nouveau sur ces populations bactériennes dénommées *small colony variants* (SCV). Les SCV ne sont pas l'apanage de *S. aureus* et ont été décrits chez d'autres espèces bactériennes impliquées dans les IOA, comme les staphylocoques à coagulase négative, *Escherichia coli* ou *Pseudomonas aeruginosa* (Von Eiff *et al.*, 2006). L'émergence de SCV résulte d'une adaptation des populations bactériennes à la pression de sélection spécifique de l'infection chronique, comme la baisse des ressources et le stress provoqué par la réponse cellulaire et/ou immunitaire. Plus spécifiquement, le phénotype SCV est particulièrement adapté à la survie intracellulaire prolongée (Tuchscherr *et al.*, 2010). Les bactéries présentant ce phénotype sont capables de persister dans le milieu intracellulaire en minimisant la réponse de défense de la cellule-hôte. Elles possèdent également une capacité accrue à envahir de nouvelles cellules par rapport aux bactéries présentant un phénotype sauvage. Leur rôle dans l'établissement d'une infection intracellulaire chronique, bien qu'encore débattu, pourrait être modélisé en trois étapes : i) parmi les bactéries impliquées dans l'infection, une sous-population gagne le milieu intracellulaire et développe un phénotype SCV ; ii) cette sous-population persiste dans un état viable, à l'abri de la destruction par la réponse immunitaire et les antibiotiques éventuellement administrés ; et iii) à la mort de la cellule-hôte, les SCV sont relargués dans le milieu extracellulaire et gagnent le cytoplasme de nouvelles cellules grâce à une capacité d'invasion accrue, établissant ainsi un réservoir bactérien pérenne, potentiellement source d'infection récidivante (Tuchscherr *et al.*, 2010).

En outre, les SCV présentent souvent une résistance accrue à différents antibiotiques. Leur vitesse de croissance ralentie diminue l'action des antibiotiques ciblant la paroi, comme dans le cas des bactéries se présentant sous forme de

biofilm. Une seconde caractéristique spécifique des SCV est une interruption du transport trans-membranaire d'électrons. Cette interruption a pour conséquence de diminuer la pénétration des antibiotiques, comme les aminosides, qui utilisent le gradient électrochimique membranaire pour traverser la membrane bactérienne et atteindre leur cible (Von Eiff *et al.*, 2006).

Enfin, les SCV chez *S. aureus* posent une difficulté diagnostique aux microbiologistes : leur métabolisme lent négative certains tests phénotypiques utilisés pour l'identification bactérienne, comme les tests d'agglutination ou les galeries d'identification biochimique, et retarde la positivité d'autres tests comme la détection de coagulase libre ou la pigmentation des colonies de *S. aureus*. Ces particularités font courir le risque d'une erreur diagnostique, ces SCV pouvant être méconnus ou identifiés à tort comme des staphylocoques à coagulase négative. Les isolats suspects de présenter ce phénotype SCV, particulièrement dans un contexte d'infection chronique, doivent idéalement faire l'objet d'une confirmation d'espèce par une méthode non phénotypique comme la détection par PCR de gènes spécifiques de *S. aureus* (Von Eiff *et al.*, 2006).

III.G. Les biofilms bactériens

L'état des bactéries libres non adhérentes est qualifié de vie planctonique, par opposition à la vie en biofilm composé d'une communauté bactérienne sédentaire au sein de laquelle les bactéries adhèrent à un substrat extracellulaire, à une surface inerte, et/ou entre elles (Figure 12). La matrice extracellulaire du biofilm dont la composition varie en fonction des espèces bactériennes présentes, est formée de différentes classes de macromolécules incluant des protéines, des polysaccharides, de l'ADN et de l'ARN. Au sein de ce biofilm, le métabolisme des bactéries est altéré et leur croissance est ralentie. L'épaisseur du biofilm est variable, et peut aller d'une monocouche bactérienne à une épaisse communauté multi-dimensionnelle. L'analyse structurale des biofilms a dévoilé une architecture complexe, dont une caractéristique majeure est la présence d'un réseau de canaux qui permettent

l'accès aux nutriments du milieu extérieur depuis les zones profondes de la communauté (Costerton *et al.*, 1995).

La majorité des bactéries impliquées dans les IOA, tout particulièrement *S. aureus* mais également les staphylocoques à coagulase négative, les entérobactéries et *P. aeruginosa*, sont capables de d'adopter ce mode de vie. Une caractéristique physiopathologique commune aux différentes formes d'IOA chroniques est la présence de matériel inerte, endogène (séquestres osseux) ou exogène (matériel prothétique), susceptible de permettre le développement d'un biofilm.

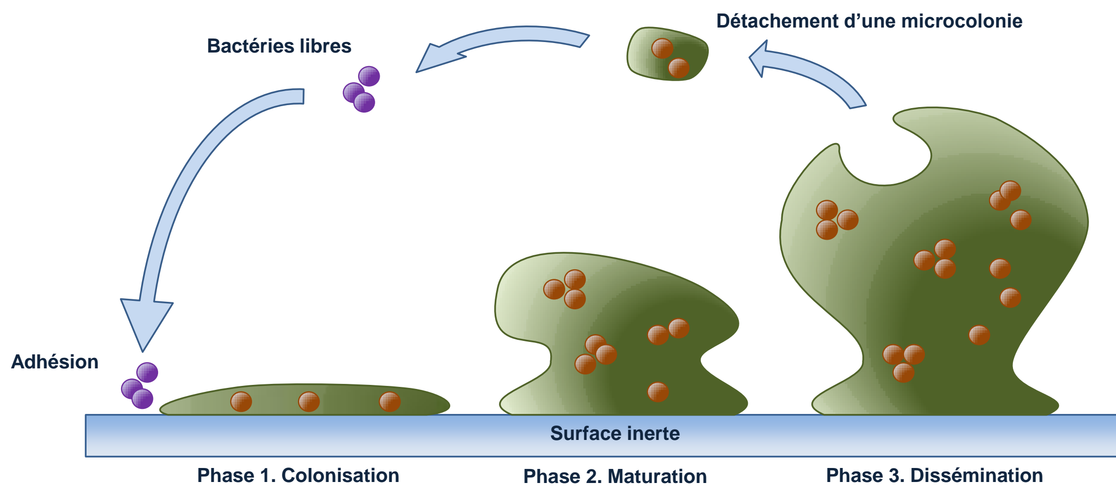

Figure 12. Cycle de vie d'un biofilm. Phase 1 : les bactéries libres, ou planctoniques, adhèrent à une surface inerte et sécrètent une matrice extracellulaire. Phase 2 : la communauté en biofilm acquiert une architecture tridimensionnelle comprenant un réseau de canaux qui permet l'acheminement des nutriments vers les zones profondes. Les bactéries ont un métabolisme ralenti et sont protégées du système immunitaire de l'hôte et de la plupart des antibiotiques. Phase 3 : des microcolonies ou des bactéries libres se détachent de la surface du biofilm. Elles dérivent dans le milieu extérieur et peuvent coloniser une nouvelle surface.

Le passage de la vie planctonique à la vie en biofilm confère plusieurs bénéfices adaptatifs. Le premier résulte de la capacité de la matrice polymérique à collecter et concentrer les nutriments provenant de l'environnement extérieur comme le carbone, l'azote et le phosphate (Beveridge *et al.*, 1997). Le deuxième bénéfice du biofilm est qu'il combine plusieurs mécanismes de résistance aux stratégies d'élimination mécaniques, immunitaires ou chimiques : sur le plan mécanique, l'adhérence et la structure flexible d'un biofilm lui permettent de résister à l'arrachement sous l'effet d'un fluide circulant (contrainte de cisaillement exercée par exemple par la circulation sanguine ou un lavage au cours d'un abord chirurgical) ; sur le plan immunitaire, la matrice polymérique du biofilm joue un rôle de bouclier contre les cellules phagocytiques de l'hôte et les molécules antimicrobiennes au sens large comme les anticorps, les protéases et les radicaux libres. Enfin, sur le plan chimique, la vie en biofilm confère aux bactéries une résistance accrue à de nombreux antibiotiques et antiseptiques, à la fois pour des raisons métaboliques et structurales (Xu *et al.*, 2000). D'une part, la vitesse de croissance et de division très ralentie des bactéries vivant en biofilm augmente leur résistance aux antibiotiques ciblant la paroi bactérienne au cours du cycle cellulaire comme les bêta-lactamines.

D’autre part, la matrice polymérique d’un biofilm a la capacité de ralentir ou empêcher la diffusion des antibiotiques jusqu’aux bactéries elles-même (De Beer *et al.*, 1994). Au final, la concentration d’antibiotique requise pour inhiber la croissance des bactéries vivant en biofilm peut être jusqu’à 1000 fois supérieure à la CMI habituellement mesurée sur les bactéries planctoniques (Schwank *et al.*, 1998); de plus, la résistance des biofilms à certaines molécules comme la vancomycine s’accroît avec leur durée d’évolution (Monzon *et al.*, 2002).

Le troisième avantage du biofilm est son potentiel de dissémination. Des microcolonies bactériennes peuvent se détacher de la surface du biofilm, soit sous la contrainte mécanique de la circulation sanguine, soit par un processus de détachement actif (Boyd et Chakrabarty, 1994). Les bactéries ainsi libérées dans le milieu extérieur peuvent dériver jusqu’à une nouvelle surface, la coloniser et établir à distance un nouveau biofilm dans une zone auparavant saine. Le mode de vie en biofilm maintient ainsi un réservoir bactérien résilient contre le système immunitaire et les antibiotiques, tout en assurant simultanément la possibilité d’une dissémination bactérienne retardée.

III.H. Impact de l’internalisation, du biofilm et des SCV sur la thérapeutique des IOA chroniques

La catégorisation d’une population bactérienne sensible ou résistante à un antibiotique donné repose actuellement sur des tests in vitro qui évaluent son activité vis-à-vis de bactéries planctoniques. Cependant, cette activité peut rarement être extrapolée aux bactéries vivant en biofilm, et de façon plus générale aux bactéries dont la croissance est ralentie. De plus, le tissu osseux n’est pas propice à la diffusion des antibiotiques. Les molécules utilisables dans les IOAs, dont le choix peut déjà être réduit du fait de taux de résistance croissants, doivent donc contourner ces écueils et garantir à la fois une bonne diffusion osseuse et une activité bactéricide sur des bactéries à croissance ralentie, éventuellement en position

intracellulaire (Lew et Waldvogel, 2004). Il est important de noter que l'activité des traitements de référence des IOA que sont les β-lactamines et les glycopeptides semble altérée dans ce contexte. La rifampicine satisfait ces contraintes et son indication est indiscutable dans la prise en charge des IOAs, à condition d'être associée à une autre molécule bactéricide comme une fluoroquinolone pour prévenir l'apparition de mutants résistants (Lew et Waldvogel, 2004). D'autres antibiotiques comme le linézolide, la daptomycine et la tigécycline ont également une place à discuter. Bien que leur utilisation dans les IOA sorte du cadre de leur autorisation de mise sur le marché, plusieurs études cliniques sont en faveur de leur efficacité dans ce contexte (Esposito et Leone, 2008). Ces molécules ont une diffusion osseuse satisfaisante, et des études menées *in vitro* ont montré qu'elles conservaient une activité intéressante vis-à-vis des bactéries en biofilm. Cependant, l'antibiothérapie de choix dans les IOA chroniques ne fait pas l'objet d'un consensus international. Enfin, la résilience des biofilms vis-à-vis des méthodes d'éradication mécanique et chimiques rendent toujours souhaitable, lorsque cela est possible, le retrait de leur surface d'attachement par une prise en charge chirurgicale visant à supprimer tout corps étranger ou inerte, qu'il s'agisse de matériel prothétique ou de séquestres osseux.

IV. SPECIFICITES DES IOA A CA-MRSA

Les *S. aureus* résistants à la méticilline (*methicillin-resistant* S. aureus*,* MRSA) représentent un des enjeux majeurs de santé publique des dernières décennies. Les MRSA constituent, depuis leur émergence, une cause d'infection importante chez les patients les plus fragiles et, dans certains services, sont devenus plus fréquents que leurs homologues sensibles (*methicillin-susceptible* S. aureus, MSSA) (National Nosocomial Infections Surveillance (Nnis), 2004). Aux Etats-Unis, le nombre de morts imputables aux MRSA dépasse depuis 2005 le nombre de morts imputables au HIV (Klevens *et al.*, 2007). Le fardeau que font peser les MRSA dans les pays à haute prévalence n'est pas seulement mesurable en termes de morbidité et de mortalité, mais également en termes économiques : toujours aux Etats-Unis, le coût global de prise en charge d'une IOA sur prothèse à MRSA s'élève à 100 000 $US, contre 70 000 $US pour une IOA à MSSA (Parvizi *et al.*, 2010).

Par-delà leur rôle dans les infections nosocomiales, les MRSA sont devenus, dans certaines régions du monde, une cause fréquente d'infection chez des patients sans antécédents, ne présentant aucun des facteurs de risque de MRSA traditionnellement reconnus. Les souches de MRSA responsables de ces infections sont distinctes, à la fois épidémiologiquement et génétiquement, des souches de MRSA nosocomiales, et ont été désignés sous le terme de *community-acquired* MRSA (CA-MRSA) (Centers for Disease Control and Prevention, 1999). La rapidité avec laquelle certains CA-MRSA se sont installés de façon endémique dans différentes régions suggère que ces souches présentent à la fois une capacité de colonisation et une virulence accrues par-rapport aux autres souches de *S. aureus*. Depuis le début des années 2000, les CA-MRSA sont devenus la première cause d'infection cutanée aux Etats-Unis, et une cause majeure d'infections invasives comme les pneumonies et les infections ostéo-articulaires (Klevens *et al.*, 2007). De plus, les CA-MRSA s'immiscent dans de nouvelles niches écologiques et sont désormais fréquents dans certains hôpitaux où ils deviennent la cause d'infections

nosocomiales comme des bactériémies ou des infections de site opératoire (ISO) (Gonzalez *et al.*, 2006a; Park *et al.*, 2009).

IV.A. L'émergence des CA-MRSA

Les MRSA ont été identifiés pour la première fois au début des années 1960 (Jevons *et al.*, 1963). Leur prévalence dans le milieu hospitalier s'est accrue au cours des années 1980, et ils sont désormais endémiques dans une majorité d'hôpitaux, particulièrement dans les services de réanimation. Alors que la résistance à la pénicilline est fréquente à la fois chez les souches communautaires et nosocomiales, la résistance à la méticilline était considérée comme un trait exclusif des souches hospitalières. Jusqu'à l'émergence des CA-MRSA, les patients atteints d'infection à MRSA présentaient des facteurs de risque bien identifiés : (i) un antécédent dans l'année précédente d'hospitalisation, dialyse, chirurgie ou une résidence dans un service de long séjour ; (ii) la présence de matériel implanté percutané comme une voie veineuse centrale ou un cathéter d'alimentation parentérale ; (iii) un antécédent d'infection à MRSA (Naimi *et al.*, 2003). De plus, l'infection à MRSA répondait à la définition de l'infection nosocomiale, dont la survenue survient après un délai de 48h par rapport à l'admission. Les MRSA isolés de patients présentant ces facteurs de risque sont maintenant désignés, d'un point de vue clinique, par le terme *healthcare-associated* (HA-) MRSA (MRSA hospitaliers) dans le but de les distinguer des CA-MRSA isolés de patients ne présentant aucun de ces facteurs de risque.

Les premières descriptions de CA-MRSA ont été faites en Australie (Udo *et al.*, 1993). Vingt-cinq isolats de MRSA ont été collectés entre 1989 et 1991, dont 18 étaient issus d'infections et 7 étaient des souches de colonisation. Aucun des patients n'avait été hospitalisé dans l'année précédente en dehors de la région étudiée, et les hôpitaux de cette région présentaient une prévalence de MRSA virtuellement nulle. En conséquence, l'acquisition des MRSA chez ces patients n'a pas été considérée comme étant d'origine nosocomiale. Le typage des souches isolées a montré que la mojorité d'entre elles étaient clonales et sensibles au

chloramphénicol, à la gentamicine et au triméthoprime, autant d'antibiotiques auxquels les MRSA des hôpitaux australiens étaient classiquement résistants. Par la suite, la caractérisation moléculaire des CA-MRSA australiens a permis de montrer que la majorité de ces souches appartiennent à une lignée particulière, ST93, désignée sous le nom de clone Queensland, bien que d'autres clones désormais pandémiques comme le CA-MRSA ST8 pulsotype USA300 soient également observés dans le pays (Coombs *et al.*, 2009).

C'est cependant aux Etats-Unis que la diffusion des CA-MRSA s'est montrée la plus spectaculaire. A la fin des années 1990s, plusieurs cas d'infection à MRSA étaient décrits à Chicago chez des enfants sans facteurs de risque (Herold *et al.*, 1998), de même que quatre cas d'infections rapidement mortelles à MRSA, toujours en pédiatrie, observés dans le Middlewest américain (Centers for Disease Control and Prevention, 1999). Par la suite, différentes épidémies à MRSA ont été observées chez des prisonniers (Centers for Disease Control and Prevention, 2001; Main *et al.*, 2005), des athlètes (Centers for Disease Control and Prevention, 2003; Kazakova *et al.*, 2005) et des recrues de l'armée (Campbell *et al.*, 2004). Ces différentes épidémies présentaient plusieurs points communs, notamment des motifs de consultation le plus souvent liés à des infections de la peau et des tissus mous, l'absence des facteurs de risque habituels d'infection à MRSA, la clonalité des souches incriminées et leur fréquente sensibilité aux antibiotiques autres que les β-lactamines. Une large majorité de ces épidémies étaient dues à un unique clone de CA-MRSA, porteur de la PVL, ST8 USA300, lequel n'était pas retrouvé parmi les MRSA responsables d'infections nosocomiales, majoritairement ST5 USA100 (Mcdougal *et al.*, 2003; Diep *et al.*, 2006b; Huang *et al.*, 2006). Compte tenu de ces différences épidémiologiques par-rapport aux MRSA hospitaliers, une distinction fut alors introduite entre les CA-MRSA et les HA-MRSA. A la suite de ces épidémies initiales et nord-américaines à CA-MRSA, des cas sporadiques d'infection ont été rapportés depuis différentes régions du monde dont l'Amérique du Nord (Fridkin *et al.*, 2005; Moran *et al.*, 2006; Klevens *et al.*, 2007), l'Amérique Centrale (Velazquez-Meza *et al.*, 2004), l'Amérique du Sud (Ribeiro *et al.*, 2005), l'Europe (Vandenesch *et al.*, 2003; Tristan *et al.*, 2007a), l'Asie (Hsu *et al.*, 2005; Hsu *et al.*, 2006a; Hsu *et al.*, 2006b), l'Océanie (Nimmo *et al.*, 2000; Munckhof *et al.*, 2003) et l'Afrique (Ramdani-Bouguessa *et al.*, 2006; Kechrid *et al.*, 2010).

IV.B. Physiopathologie des IOA à CA-MRSA

Les formes cliniques d'IOA à *S. aureus*, qu'il s'agisse de MSSA ou de HA-MRSA, sont variées et hétérogènes. En comparaison, les IOA à CA-MRSA représentent un groupe homogène d'entités cliniques. Contrairement aux IOA à MSSA et à HA-MRSA, les IOA à CA-MRSA touchent majoritairement les enfants, et la présentation initiale est toujours aiguë (Martinez-Aguilar *et al.*, 2004). Les enfants atteints d'IOA à CA-MRSA se présentent le plus souvent avec une ostéomyélite hématogène du tibia ou du fémur, ou une arthrite septique du genou, de la hanche ou de l'épaule. Il est important de préciser que les souches de MSSA portant, comme les CA-MRSA, les gènes *pvl* codant la leucocidine de Panton-Valentine (PVL), sont à l'origine de tableaux d'IOA très comparables à ceux des CA-MRSA (Dohin *et al.*, 2007), et que la majorité des IOA à *S. aureus pvl*+ en Europe de l'Ouest dont la France sont le fait de MSSA *pvl*+ plutôt que de CA-MRSA.

IV.B.1. Voies de contamination, présentation clinique et pronostic

De la même façon que les ostéomyélites de l'enfant à MSSA, les IOA à CA-MRSA sont classiquement d'origine hématogène. L'origine de la bactériémie est évidente lorsqu'une infection de la peau et des tissus mous est présente ou a précédé l'IOA, mais elle peut être absente. La métaphyse des os longs est le site le plus fréquemment touché lors des IOA à CA-MRSA en pédiatrie. Les études épidémiologiques menées aux Etats-Unis depuis l'émergence des CA-MRSA ont montré que la dissémination de ces souches s'était accompagné d'une augmentation non seulement d'incidence, mais aussi de sévérité des IOA (Gwynne-Jones et Stott, 1999; Martinez-Aguilar *et al.*, 2004; Gonzalez *et al.*, 2005; Arnold *et al.*, 2006; Gerber *et al.*, 2009). Les enfants atteints d'IOA à CA-MRSA, par comparaison avec d'autres fonds génétiques de *S. aureus*, présentent une réponse inflammatoire plus aiguë (Bocchini *et al.*, 2006), des durées d'hospitalisation et de fièvre plus élevées (Martinez-Aguilar *et al.*, 2004; Arnold *et al.*, 2006), et nécessitent plus souvent le recours à la chirurgie (Arnold *et al.*, 2006) et/ou l'admission en soins intensifs

(Gonzalez *et al.*, 2005; Vander Have *et al.*, 2009). Enfin, les IOA à CA-MRSA sont très souvent grevées de complications musculo-squelettiques comme des abcès sous-périostés ou musculaires, une thrombose veineuse, une fasciite nécrosante ou un choc septique (Martinez-Aguilar *et al.*, 2004; Arnold *et al.*, 2006; Bocchini *et al.*, 2006; Gonzalez *et al.*, 2006b).

IV.B.2. Déterminants de virulence spécifiques des CA-MRSA

Les facteurs d'adhésion et d'invasion, dont les FnBP, classiquement impliqués dans la physiopathologie des IOA à *S. aureus* sont présents chez les CA-MRSA (Baba *et al.*, 2002; Stefani *et al.*, 2009), mais la capacité d'invasion et de survie intracellulaire de ces derniers n'a jamais été étudiée. En plus des mécanismes classiques de virulence de *S. aureus*, les CA-MRSA possèdent plusieurs déterminants pathogéniques spécifiques, dont la PVL (Vandenesch *et al.*, 2003), les *staphylococcal cassette chromosome* mec (SCC*mec*) de types IV à VII (Baba *et al.*, 2002), et présentent une surexpression de l'α-toxine et des *phenol-soluble modulins* (PSM) (Wang *et al.*, 2007; Montgomery *et al.*, 2010).

IV.B.2.a. La leucocidine de Panton-Valentine

La PVL est une exotoxine protéique de la classe des PFT, initialement décrite en 1894 par Van de Velde qui démontra sa capacité à lyser les leucocytes et la désigna sous le terme de « *substance leukocidin* » (Boyle-Vavra et Daum, 2007). Panton et Valentine ont, en 1932, établi l'association entre cette leucocidine et les infections de la peau et des tissus mous. Par la suite, l'identification des gènes codant cette toxine a permis d'établir le lien épidémiologique entre les souches *pvl*+ et la survenue de pneumonies nécrosantes, tout d'abord chez les MSSA (Lina *et al.*, 1999; Gillet *et al.*, 2002), puis chez les CA-MRSA (Vandenesch *et al.*, 2003). Les résultats de nombreuses études épidémiologiques ont ensuite renforcé l'association entre la PVL et les CA-MRSA, et cette toxine est considérée à ce jour comme un déterminant de virulence majeur de ces souches, et non pas comme un simple marqueur moléculaire (Vandenesch *et al.*, 2003; Tristan *et al.*, 2007b). Il est

important de noter que, bien que *S. aureus* soit doté d'un large répertoire de gènes transférables et codant des toxines, les gènes codant la PVL sont ceux dont la présence chez les CA-MRSA est la plus courante (Kaneko et Kamio, 2004; Miller *et al.*, 2005; Diep *et al.*, 2006a).

La PVL appartient à la famille des leucocidines à deux composants, ou toxines synergohyménotropes (Kaneko et Kamio, 2004). Ces toxines se composent de deux polypeptides, les composants S (slow) et F (fast) désignés ainsi sur la base de leur vitesse d'élution en chromatographie à échange d'ions. Les deux composants LukF-PV et LukS-PV de la PVL sont codés par l'opéron *luk*PV, plus communément désigné opéron *pvl*. Cet opéron est porté par différents phages transférables, ΦSLT, ΦPVL, ΦSA2MW ou ΦSA2usa (Szmigielski *et al.*, 1999; Diep *et al.*, 2006b; Ma *et al.*, 2006). Les deux composants LukS-PV et LukF-PV sont sécrétés ; le premier se fixe à un récepteur encore non identifié à la surface des cellules de la lignée myéloïde et se dimérise alors avec le second. De nouveaux composants LukS-PV et LukF-PV se lient alors séquentiellement au dimère jusqu'à former un assemblage heptamérique en tonneau beta qui réalise un pore traversant la membrane plasmique. Ce mécanisme physiopathologique est similaire à celui de l'α-toxine ; cependant, l'α-toxine présente une spécificité de cible très différente et ne lyse pas les polynucléaires neutrophiles (Kaneko et Kamio, 2004; Joubert *et al.*, 2007). Les déterminants moléculaires de la spécificité de cible de ces deux toxines pourtant structurellement similaires ne sont pas élucidés (Valeva *et al.*, 1997).

En fonction de la concentration appliquée, la PVL peut causer l'apoptose ou la lyse des leucocytes. L'apoptose a été associée à la formation de pores dans la membrane mitochondriale (Genestier *et al.*, 2005). Après sa liaison à la surface des leucocytes, LukS-PV est phosphorylé par les protéines kinases A ou C de l'hôte (Kaneko et Kamio, 2004). Après fixation de la PVL, une activation des canaux calciques est observée chez la cellule cible ; cette activation suggère l'induction de voies de signalisation associées à la production de médiateurs de l'inflammation. En effet, la PVL, lorsqu'elle est appliquée à des concentrations sub-lytiques, induit chez les polynucléaires neutrophiles la sécrétion d'espèces réactives de l'oxygène et de composés chimiotactiques, aboutissant au final à une exacerbation de la réponse inflammatoire locale et de l'infiltratrion leucocytaire (Konig *et al.*, 1994; Konig *et al.*,

1995; Colin et Monteil, 2003). Bien que la lyse et l'apoptose des polynucléaires neutrophiles sont clairement impliqués dans la pathogénie de la PVL, les mécanismes associés à la nécrose tissulaire locale et au sepsis sévère ne sont pas complètement élucidés. En effet, la PVL purifiée n'a aucun effet sur des cellules épithéliales en culture, il est donc peu probable que cette toxine soit directement responsable de nécrose tissulaire (De Bentzmann *et al.*, 2004). Le modèle le plus probable est celui dans lequel la nécrose tissulaire et les manifestations systémiques liées à la PVL sont médiées indirectement par le recrutement des leucocytes, leur lyse au site de l'infection, le relargage de leur contenu cytotoxique, et enfin la cascade inflammatoire déclenchée et entretenue par ce relargage (Figure 13) (Boyle-Vavra et Daum, 2007).

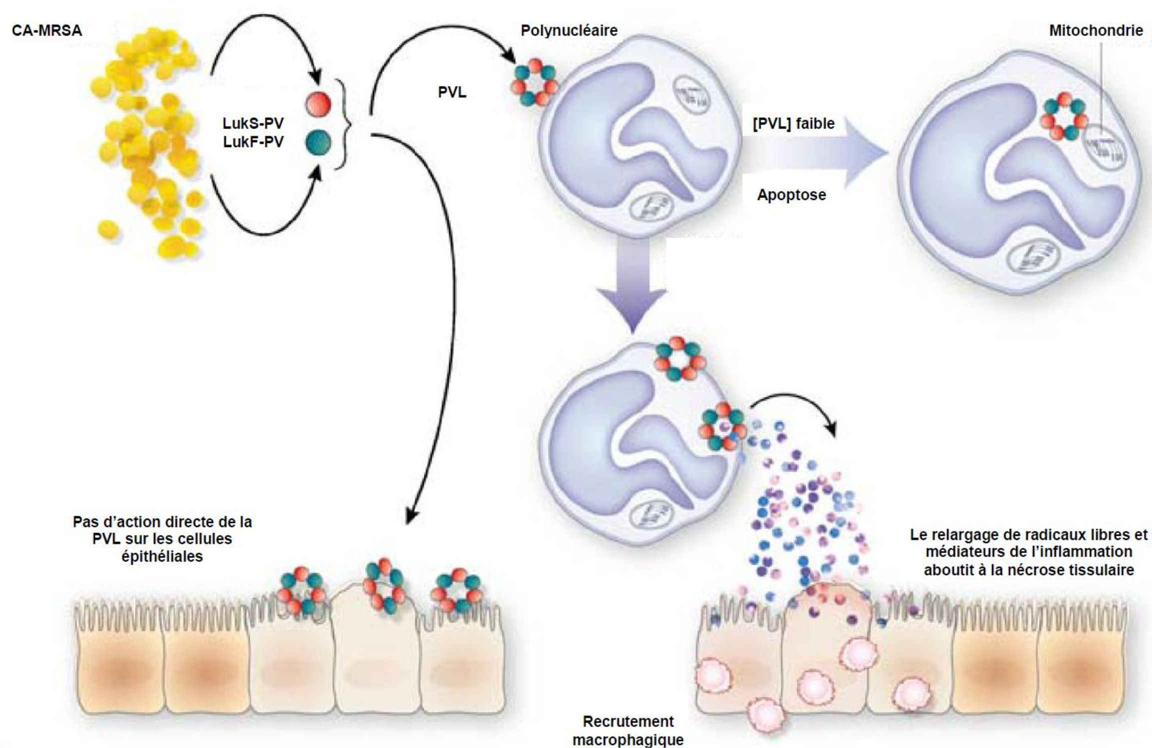

Figure 13. Modèle d'action de la PVL aboutissant à la nécrose tissulaire. Les deux composants LukS-PV et LukF-PV de la PVL s'assemblent sous forme d'heptamères à la surface des polynucléaires et créent des pores dans la membrane plasmique. A faible concentration, la PVL induit l'apoptose des polynucléaires en ciblant les mitochondries (Genestier *et al.*, 2005). A forte concentration, la lyse des polynucléaires par la PVL relargue leur contenu cytotoxique comme les radicaux libres et aboutit à la nécrose tissulaire locale. Ce relargage déclenche et entretient une réponse inflammatoire locale qui participe également à la nécrose tissulaire. D'après (Boyle-Vavra et Daum, 2007).

Le rôle pathogénique de la PVL a été investigué dans différents modèles in vitro et in vivo. In vitro, la PVL lyse les polynucléaires humains et cuniculicoles alors que les polynucléaires murins sont nettement moins sensibles (Szmigielski *et al.*, 1966; Grojec et Jeljaszewicz, 1981; Szmigielski *et al.*, 1999). La PVL purifiée, injectée par voie intraveineuse chez le lapin, induit une déplétion transitoire en polynucléaires suivie d'une polynucléose majeure, mais n'est pas létale (Szmigielski *et al.*, 1966). La PVL purifiée cause également chez le lapin des lésions dermo-nécrotiques de taille dose-dépendante (Ward et Turner, 1980; Szmigielski *et al.*, 1999). Chez la souris, l'instillation nasale de PVL purifiée cause des lésions pulmonaires nécrotiques aboutissant à la mort (Labandeira-Rey *et al.*, 2007). Chez l'homme, la PVL est sécrétée à des niveaux toxiques dans les abcès (Badiou *et al.*, 2008). Enfin, en modèle d'ostéomyélite chez le lapin, la souche CA-MRSA USA300 LAC *pvl*+ induit significativement plus d'abcès péri-osseux et musculaires que son mutant isogénique LACΔ*pvl* (Cremieux *et al.*, 2009). L'ensemble de ces données a permis d'établir que la PVL est impliquée dans la nécrose tissulaire locale, y compris au cours des IOA, à cause de son action indirecte de recrutement et lyse des polynucléaires neutrophiles et des monocytes-macrophages.

IV.B.2.b. Eléments SCC*mec* associés aux CA-MRSA

La résistance aux β-lactamines chez les MRSA est codée par le gène *mec*A, lequel est porté par un élément génétique mobile, le *staphylococcal cassette chromosome* mec (SCC*mec*) dont existent plusieurs allotypes. La présence des SCC*mec* de type I à III chez les HA-MRSA a classiquement été associée à un coût biologique chez ces souches, lié d'une part à l'entretien et à la réplication de cet élément de grande taille (environ 70 kb) et d'autre part à l'expression des différents déterminants de résistance qu'il contient, outre *mec*A. Chez les CA-MRSA cependant, le gène *mec*A est porté par des éléments SCC*mec* de types différents ; le type IV est majoritaire, mais les types V à VII sont également observés. SCC*mec* IV, caractéristique des premiers CA-MRSA, a été retrouvée chez des souches de *S. epidermidis* isolées dans les années 1970, alors qu'aucun MRSA de cette période ne portait cette cassette. Il a donc été proposé que SCC*mec* IV provient de

S. epidermidis (Barbier *et al.*, 2010). SCC*mec* IV est retrouvée dans de nombreux fonds génétiques différents ; elle représente la moitié des événements de transfert de SCC*mec* chez *S. aureus* (Robinson et Enright, 2003; Feng *et al.*, 2008). Elle semble conférer un avantage sélectif majeur, comme en témoigne l'exemple du ST30 lysotype 80/81, clone de SASM PVL positif quasiment disparu dans les années 1960 à l'introduction de la méticilline, qui a pu réémerger après l'acquisition de SCC*mec* IV jusqu'à redevenir pandémique en tant que CA-MRSA (Robinson *et al.*, 2005).

Les principales hypothèses avancées pour expliquer ce succès de SCC*mec* IV se basent sur sa petite taille (20 kb) et son contenu restreint en gènes de résistance par rapport aux SCC*mec* des SARM-H. Cette taille réduite pourrait permettre à cet élément d'être transféré plus efficacement, notamment par transduction. Des comparaisons de fitness entre différentes souches isogéniques complémentées par différents allotypes de SCC*mec* ont permis de montrer que SCC*mec* I, typique des HA-MRSA, impose un coût biologique significatif à son hôte en termes de vitesse de croissance in vitro et de consommation d'ATP, alors que le coût biologique de SCC*mec* IV est indétectable (Lee *et al.*, 2007). De plus, une souche sauvage de CA-MRSA USA300 portant SCC*mec* IV présentait le même pouvoir invasif que son mutant isogénique ∆SCC*mec* dans un modèle de co-infection chez le lapin (Diep *et al.*, 2008). De plus, certaines cassettes SCC*mec* des HA-MRSA sont porteuses d'un gène, désigné *fudoh*, associé à une perte de virulence (Kaito *et al.*, 2008). L'ensemble de ces observations indique que SCC*mec* IV impose aux CA-MRSA un coût biologique minimal voire nul, alors que les HA-MRSA restent dépendants d'une forte pression de sélection antibiotique pour contrebalancer le coût biologique imposé par les multiples déterminants de résistance portés par les SCC*mec* I à III (Diep et Otto, 2008). Cependant, la ségrégation des types SCC*mec* entre CA-MRSA et HA-MRSA tend actuellement à s'estomper avec la diffusion de clones de HA-MRSA *pvl*- porteurs de SCC*mec* de type IV ou apparenté comme le clone ST8-Lyon ou le clone ST22-Barnim (Dauwalder *et al.*, 2008; Grundmann *et al.*, 2010).

IV.B.2.c. Surexpression de l'α-toxine et des phenol-soluble modulins chez les CA-MRSA

La pathogénie des CA-MRSA n'est pas seulement associée à la présence dans leur génome de déterminants de virulence portés par des éléments génétiques mobiles comme la PVL. Certaines toxines appartenant au génome cœur de *S. aureus*, et virtuellement présentes chez l'ensemble des souches de l'espèce, sont exprimées à de plus hauts niveaux chez les CA-MRSA par rapport aux autres fonds génétiques (Otto, 2010). Cette surexpression a été documentée pour l'α-toxine, dont le rôle a été décrit ci-dessus, mais également pour les phenol-soluble modulins (PSMs). Bien que les PSMs appartiennent au génome cœur de *S. aureus*, c'est chez une souche de CA-MRSA USA300 qu'ils ont été décrits initialement (Wang *et al.*, 2007). Ces peptides de petite taille (entre 20 et 30 AA) sont codés par des cadres de lecture dont la faible longueur explique qu'ils n'aient pas été détectés par les analyses génomiques antérieures à leur découverte. Leur structure en hélice alpha et leur caractère fortement amphipathique leur confère une action surfactante qui leur permet probablement de déstabiliser les membranes lipiques (Wang *et al.*, 2007). Leur implication dans la pathogénie des CA-MRSA a été démontrée à la fois par des approches de perte et de gain de fonction : l'inactivation des gènes codant les PSM induit une perte de virulence chez les CA-MRSA USA300, alors qu'à l'inverse la surexpression de PSM chez la souche HA-MRSA MRSA252 s'accompagne d'une augmentation de virulence (Wang *et al.*, 2007). Les PSM sont capables in vitro de provoquer la lyse des leucocytes (Figure 14), ainsi que d'induire leur recrutement en exerçant un pouvoir chimiotactique via le *formyl peptide receptor* 2 (FPR2) des polynucléaires neutrophiles (Wang *et al.*, 2007; Kretschmer *et al.*, 2010; Rautenberg *et al.*, 2011). Ce rôle des PSM dans la virulence des CA-MRSA semblerait donc très proche de celui de la PVL. Ces propriétés des PSM ont été investiguées dans des modèles in vitro et en conditions contrôlées ; il a par la suite été montré que les pouvoirs lytique et chimiotactique des PSM étaient abolis par les composants du sérum (Figure 15) (Surewaard *et al.*, 2012). La pertinence clinique des PSM en tant que toxines à action distante comme la PVL a donc été remise en question ; les mêmes auteurs, ayant observé une probable sécrétion de PSM par des *S. aureus*

après phagocytose par les polynucléaires neutrophiles, ont proposé que le pouvoir lytique des PSM pourrait en revanche prendre place au niveau intracellulaire.

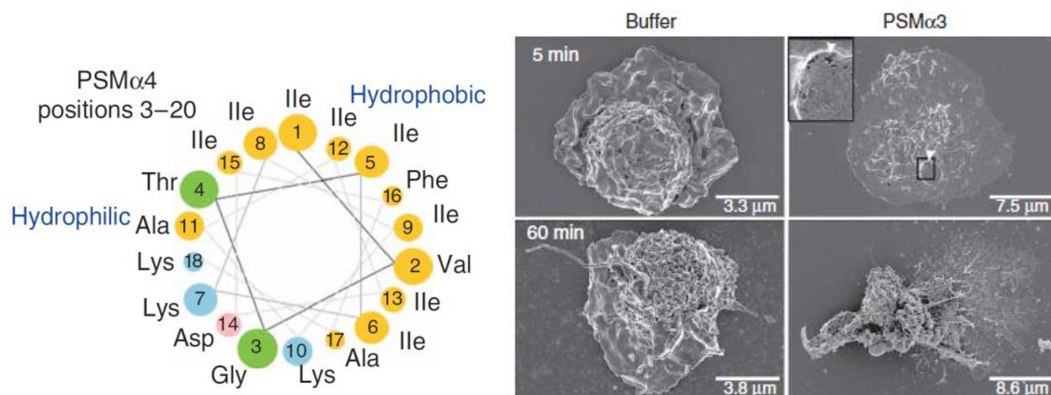

Figure 14. Structure et propriétés lytiques des PSM. A : modélisation de la structure en hélice alpha des PSM et mise en évidence de leur caractère amphipathique ; B : Lyse des polynucléaires neutrophiles par un PSM de synthèse, observée en microscopie électronique à balayage. Les flèches indiquent les zones interprétées par les auteurs comme des trous dans la membrane plasmique du polynucléaire. D'après (Wang *et al.*, 2007).

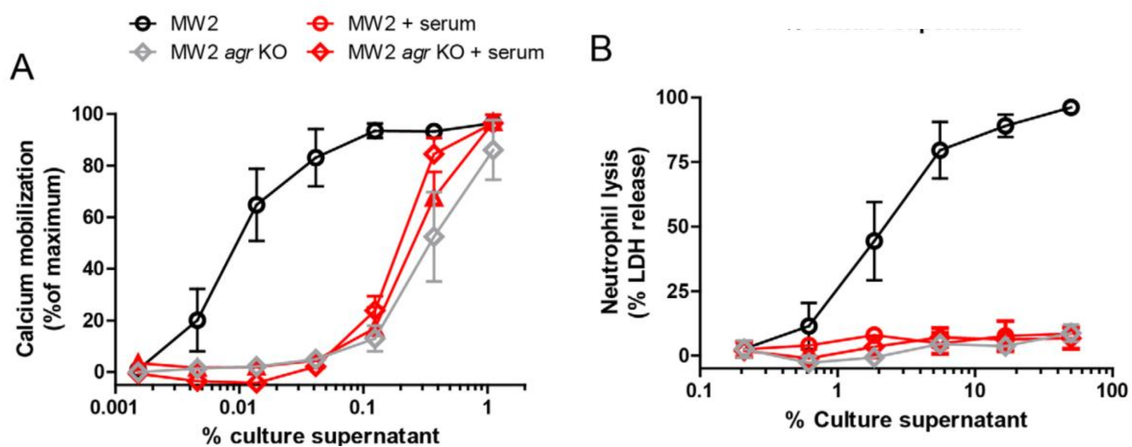

Figure 15. Le sérum inhibe les propriétés lytique et chimiotactique des PSM. A : l'adjonction de sérum au surnageant de culture de la souche de CA-MRSA MW2 inhibe l'afflux calcique FPR2-dépendant chez les cellules HL-60 transfectées par FPR2. Le surnageant de la souche MW2 sauvage mélangé au sérum déclenche un afflux calcique similaire au surnageant de la souche MW2 Δ*agr* ne contenant pas de PSM ; B : le pouvoir lytique du surnageant de culture de MW2 sur les polynucléaires neutrophiles est aboli par l'adjonction de sérum. D'après (Surewaard *et al.*, 2012).

V. CONCLUSIONS DE LA PARTIE BIBLIOGRAPHIQUE

Au cours de cette synthèse bibliographique, nous avons pu mettre en lumière que les difficultés de traitement des IOA à *S. aureus* et la perte de tissu osseux associée à ces infections relèvent de mécanismes physiopathologiques multi-factoriels, dont certains sont de découverte relativement récente et sont encore imparfaitement compris. L'invasion des ostéoblastes par *S. aureus* est probablement un des facteurs majeurs du caractère chronique des IOA. Nous avons vu que les souches de *S. aureus* peuvent présenter des capacités variables en termes d'invasion cellulaire, de survie intracellulaire et d'induction de la mort de la cellule hôte. Ces observations nous ont amené à poser la question suivante : si les interactions entre ostéoblastes et *S. aureus* sont dépendantes du fond génétique de la souche, peut-on observer un lien entre la nature de ces interactions (capacité d'invasion, survie bactérienne, destruction de l'ostéoblaste) et la présentation clinique de l'IOA ?

Dans ce contexte, les CA-MRSA et les HA-MRSA fournissent un modèle d'étude précieux à plusieurs titres. D'une part, la gravité exceptionnelle des IOA à CA-MRSA justifie à elle seule l'exploration de leurs interactions avec les ostéoblastes. En effet, la présence de mécanismes de pathogénie ciblant les ostéoblastes permettrait de désigner des cibles thérapeutiques éventuelles, dédiées aux CA-MRSA. D'autre part, le caractère quasiment systématiquement hypervirulent des clones majeurs de CA-MRSA, en comparaison aux HA-MRSA, encourage à rechercher des différences de virulence entre ces pathogènes au niveau cellulaire. Le faible nombre de clones circulants de CA-MRSA et de HA-MRSA rend envisageable l'obtention de conclusions fiables basées sur un nombre restreint de souches : en sélectionnant des souches représentatives des principales lignées de MRSA, nous considérons que les conclusions ainsi obtenues peuvent raisonablement être extrapolées à l'ensemble des MRSA. En revanche, la forte diversité génétique des MSSA rend difficilement atteignable l'obtention de

conclusions généralisables à ce groupe, qui nécessiteraient l'étude d'un nombre très élevé de souches.

Nous avons vu également que les principaux facteurs bactériens responsables de l'invasion cellulaire sont les protéines de surface FnPB. Ces adhésines sont, comme d'autres protéines de surface de *S. aureus*, sous le contrôle des systèmes de régulation, dont *agr*, qui assurent la balance d'expression entre exotoxines et protéines de surface. De nombreux travaux ont démontré l'influence, positive ou négative, de différents antibiotiques en concentration sub-inhibitrices sur l'expression des exotoxines de *S. aureus* (Herbert *et al.*, 2001; Bernardo *et al.*, 2004; Dumitrescu *et al.*, 2007; Stevens *et al.*, 2007). Le tissu osseux est un des plus difficiles d'accès aux antibiotiques, et la survenue de telles concentrations sub-inhibitrices au cours du traitement médical des IOA est très probable. Dans ce contexte, nous avons émis l'hypothèse que certains antibiotiques, utilisés en prophylaxie ou en thérapeutique, pouvaient moduler le niveau d'expression des FnBP à la surface bactérienne, et par conséquent la capacité de la souche infectante à envahir de nouveaux ostéoblastes.

Au final, ces différentes questions et hypothèses nous ont amenés à centrer notre travail expérimental d'une part sur les interactions entre ostéoblastes, CA-MRSA et HA-MRSA, en termes de capacité d'invasion, de survie intracellulaire et de destruction des cellules infectées, et d'autre part sur la modulation de l'expression des FnBP et de l'invasion par les antibiotiques à dose sub-inhibitrice. Ces études de co-culture ont nécessité le développement d'outils spécifiques, ainsi que d'un modèle expérimental adapté à l'exploration de l'invasion des ostéoblastes par *S. aureus*.

TRAVAIL PERSONNEL

I. INTRODUCTION

La revue de la littérature présentée dans la section précédente a permis de mettre en lumière le caractère toujours problématique de la prise en charge des IOA à *S. aureus* et les principales questions soulevées à propos des interactions entre ce pathogène et les cellules osseuses, qui ont trait d'une part aux différences de virulence potentielles entre CA-MRSA et HA-MRSA lors de l'invasion des ostéoblastes, et d'autre part à l'influence des antibiotiques sur la capacité d'invasion de *S. aureus*.

L'étude de ces deux questions a nécessité en premier lieu la mise en place d'un modèle d'étude adapté. La comparaison de différentes souches cliniques ou isogéniques nécessite l'obtention de mesures fiables de la quantité de bactéries adhérentes ou internalisées, viables ou non-viables. Or, les techniques expérimentales disponibles au moment de l'initiation de nos travaux étaient notoirement peu reproductibles (Qazi *et al.*, 2001), et d'une lourdeur difficilement compatible avec l'étude d'un nombre élevé de conditions et de souches. La première partie de nos travaux a donc consisté à rechercher et optimiser différentes techniques de mesure de l'adhésion et de l'internalisation de *S. aureus*. Ces travaux ont permis de définir un protocole expérimental compatible avec une utilisation à haut débit et dont la description a donné lieu à une publication (Trouillet S, Rasigade JP, Lhoste Y *et al.,* A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells, *Journal of Microbiological Methods,* 2011). Il est cependant nécessaire de préciser que cette approche, pour des raisons techniques et stratégiques discutées dans la troisième

partie de cette thèse, n'a finalement pas été employée dans la suite de nos travaux qui ont privilégié une version optimisée du protocole de protection à la gentamicine pour quantifier la capacité d'invasion.

La seconde partie de nos travaux a été dédiée aux phénotypes d'invasion et de virulence des CA-MRSA et des HA-MRSA dans un modèle de co-culture avec les ostéoblastes humains. Ces travaux ont permis d'établir que les CA-MRSA et les HA-MRSA présentaient des différences majeures en termes de virulence vis-à-vis des ostéoblastes. Les HA-MRSA sont capables d'envahir efficacement les ostéoblastes et de survivre à l'intérieur de ces cellules sans provoquer de dommages cellulaires majeurs ; à l'inverse, si les CA-MRSA envahissent moins efficacement les ostéoblastes et survivent également moins efficacement dans le compartiment intracellulaire, ils causent d'importants dommages aux cellules infectées. En utilisant différentes approches complémentaires, à la fois populationnelles et basées sur la construction de mutants isogéniques, nous avons pu déterminer quels facteurs de virulence des CA-MRSA étaient responsables de l'induction de la mort chez les ostéoblastes infectés, ainsi que les voies de régulation associées au phénotype cytotoxique. Ces résultats ont été soumis pour publication à *PLoS Pathogens*.

Dans la troisième partie de nos travaux, nous avons étudié l'impact de différentes molécules anti-staphylococciques, appliquées à des doses sub-inhibitrices, sur la régulation de l'expression des FnBP, sur l'adhésion de *S. aureus* à la fibronectine et aux ostéoblastes, et sur la capacité d'invasion cellulaire. Nous avons pu établir que certaines molécules avaient effectivement un impact, positif ou négatif, sur l'expression des FnBP et l'adhésion à la fibronectine in vitro, mais que cet effet n'était pas corrélé à une modification du pouvoir invasif de la bactérie dans le modèle utilisé. Ces travaux ont donné lieu à une publication (Rasigade JP, Moulay A, Lhoste Y *et al.*, Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*, *BMC Microbiology*, 2011).

L'objectif global de notre travail a ainsi été d'approfondir les connaissances sur les interactions *S. aureus*-ostéoblastes, avec comme cahier des charges de dépister des mécanismes physiopathologiques pertinents dans la compréhension des IOA.

II. Première Publication : A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells

Sophie Trouillet, Jean-Philippe Rasigade, Yannick Lhoste, Tristan Ferry, François Vandenesch, Jérôme Etienne, Frédéric Laurent.

***Journal of Microbiological Methods* 2011 ; 86 : 145-149**

L'étude des interactions entre bactéries et cellules eucaryotes implique de pouvoir quantifier avec précision l'adhésion et l'invasion bactérienne. Une technique classiquement utilisée dans les modèles de culture cellulaire est la protection à la gentamicine, qui consiste, après une étape de co-culture bactéries-cellules pendant laquelle a lieu l'invasion, à ajouter dans le milieu de culture un composé bactéricide, le plus souvent la gentamicine, qui permet de tuer les bactéries extracellulaires tout en sélectionnant les bactéries intracellulaires. A l'issue de l'incubation, les cellules infectées sont lysées et le lysat est ensemencé sur gélose pour obtenir un compte de CFU. Cette méthode, outre son manque de reproductibilité, ne permet pas de quantifier l'invasion indépendamment de la survie bactérienne : seules les bactéries viables, dont la culture est visible sur gélose, peuvent être dénombrées.

Pour s'affranchir de cette limite et quantifier les bactéries internalisées indépendamment de leur viabilité, des méthodes basées sur la cytométrie en flux ont été développées. L'utilisation d'un marqueur fluorescent ciblant un antigène de *S. aureus* permet de marquer spécifiquement les bactéries intracellulaires et de quantifier d'une part la proportion de cellules infectées, et d'autre part le niveau de fluorescence par cellule comme marqueur de la biomasse bactérienne intracellulaire. Ces techniques présentent cependant une limite : le marquage des bactéries est

réalisé préalablement à l'étape d'infection, soit par construction génétique (la bactérie exprime une molécule fluorescente), soit chimiquement (la bactérie est marquée à l'aide d'anticorps). Cette approche est donc peu physiologique en ce sens que les bactéries étudiées ont subi une transformation avant leur utilisation dans le modèle d'infection : on ne peut exclure que cette transformation puisse influencer par elle-même les résultats des expérimentations.

Pour optimiser la pertinence physiologique des modèles d'infection, nous avons développé une méthode d'analyse dans laquelle le marquage des bactéries a lieu après l'étape d'infection. Les bactéries co-cultivées avec les cellules sont donc dans un état aussi physiologique que possible. Cette approche a été rendue possible grâce à l'emploi d'un fluorophore spécifique couplé à un antibiotique ciblant avec une haute affinité le peptidoglycane des bactéries Gram positif, la vancomycine.

Contribution personnelle au travail : apport de la problématique et des objectifs de la méthode, participation aux manipulations de mise au point, analyse des données, participation à la rédaction et à la révision du manuscrit.

Contributions majeures non réalisées par l'auteur : S. Trouillet a apporté son savoir-faire en cytométrie en flux, a plannifié et réalisé les manipulations de mise au point, a participé à l'analyse des données et a rédigé la première version du manuscrit.

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A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells

Sophie Trouillet [a,b,*], Jean-Philippe Rasigade [a,b], Yannick Lhoste [a,b], Tristan Ferry [a,c], François Vandenesch [a], Jerome Etienne [a], Frederic Laurent [a,b]

[a] INSERM U851, National Reference Center for Staphylococci, University of Lyon, 8 rue Guillaume Paradin, 69008 Lyon, France
[b] Department of Clinical Microbiology, Hospices Civils de Lyon, Northern Hospital Group, 103 Grande-Rue de la Croix-Rousse, 69004 Lyon, France
[c] Department of Infectious and Tropical Diseases, Hospices Civils de Lyon, Northern Hospital Group, 103 Grande-Rue de la Croix-Rousse, 69004 Lyon, France

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ABSTRACT

Flow cytometry is a powerful tool for analyzing the adhesion to and invasion of *Staphylococcus aureus* (*S. aureus*) to eukaryotic cells. Established techniques have used bacteria that have been genetically modified to express fluorescent proteins or directly labeled with fluorochromes prior to infection. Such approaches are appropriate in most cases; however, the use of genetically or chemically altered bacteria could introduce a bias when measuring fine differences in adhesion and invasiveness. Here, we describe a combined flow cytometry-based invasion and adhesion assay that does not require the processing of bacteria prior to internalization. This method was performed on osteoblastic MG-63 cells infected with *S. aureus* reference strain 8325-4 and its invasion-deficient isogenic mutant, which carries deletions in the genes encoding fibronectin-binding proteins A and B. The data from this assay were compared to those obtained using the standard gentamicin protection assay. The results obtained by the two methods were consistent. Moreover, quantification of internalized bacteria was more reproducible using the flow cytometry-based assay than the gentamicin protection assay, which allowed for the simultaneous quantification of host cell adhesion and invasion.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is the leading cause of bone and joint infection (BJI) (Berendt and Byren, 2004). The successful cure of these infections is challenging due to frequent chronicization and relapse (Stevens et al., 2007). The recurrent nature of BJIs is related to the ability of *S. aureus* to invade and survive within host cells (Jevon et al., 1999). Initially, bacteria adhere to the cell membrane by means of several surface proteins, including a group of surface adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster and Hook, 1998). Among these MSCRAMMs, the fibronectin-binding proteins (FnBPs) are necessary and sufficient for internalization of bacteria (Ahmed et al., 2001; Sinha et al., 2000). Internalization is an active process with respect to the host cell and a passive process with respect to the bacteria (Ellington et al., 1999).

Several *in vitro* models have been developed to investigate the internalization process and to quantify membrane-bound and intracellular *S. aureus*. The widely used antibiotic protection assay relies on the specific protection of intracellular bacteria from membrane-impermeable antimicrobial agents and the quantification of live bacteria by plate counting after host cell lysis (Jevon et al., 1999; Wright and Friedland, 2004). Consequently, this method is time-consuming and lacks reproducibility. More recently, high-throughput methods that use flow cytometry have been proposed. However, the methods published to date have required that the bacteria be either genetically modified to express fluorescent proteins or chemically labeled prior to the experiment (Agerer et al., 2004; DeLoid et al., 2009; Pils et al., 2006).

Here, we describe a combined flow cytometry-based invasion and adhesion assay. To mimic the physiological conditions of bacteria–cell interactions, this assay does not require the processing of bacteria prior to internalization. This method was applied to *S. aureus* reference strain 8325-4 and its invasion-defective isogenic mutant DU5883, which has deletions in the genes encoding the fibronectin-binding proteins (Δ*fnbA*/*B*), and the results were compared to the gentamicin protection assay.

Abbreviations: BJI, bone and joint infection; FCBA, flow cytometry based approach; GPA, gentamicin protection assay; VBFL, vancomycin-Bodipy FL.

* Corresponding author at: Laboratoire de Bactériologie, Centre de Biologie Nord, Groupement Hospitalier Nord, INSERM U851, Centre National de Référence des Staphylocoques, 103, Grande-Rue de la Croix-Rousse, 69004 Lyon, France. Tel.: +33 472 07 18 37; fax: +33 472 07 18 42.

E-mail addresses: trouilletsophie@aol.com (S. Trouillet), jean-philippe.rasigade@chu-lyon.fr (J.-P. Rasigade), yanlho@hotmail.com (Y. Lhoste), tristan.ferry@chu-lyon.fr (T. Ferry), francois.vandenesch@univ-lyon1.fr (F. Vandenesch), jerome.etienne@univ-lyon1.fr (J. Etienne), frederic.laurent@univ-lyon1.fr (F. Laurent).

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2. Materials and methods

2.1. Cell culture

All cell culture reagents were obtained from GIBCO (Paisley, United Kingdom). Human MG-63 osteoblastic cells (LGC Standards, Teddington, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and 25 mM HEPES and supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin (referred to here as culture medium). The cells were passaged twice a week up to passage 10 after being thawed from stock culture.

2.2. Bacterial isolates

Methicillin- and gentamicin-susceptible *S. aureus* reference strain 8325-4 and mutant strain DU5883 were a generous gift from Tim Foster (Greene et al., 1995). DU5883 is an isogenic mutant of strain 8325-4 with deletions in the *fnbA/B* genes. The bacteria were grown overnight in brain heart infusion broth. Before the internalization step, the bacteria were washed with DMEM and suspended in DMEM with 10% FBS.

2.3. Infection of MG-63 cells by S. aureus

MG-63 cells were seeded (50,000 cells/ml) into 24-well culture plates and incubated in culture medium at 37 °C in 5% CO_2 for 48 h until 80% confluence. Cells were washed twice with 1 ml of DMEM before the addition of bacteria at a multiplicity of infection (MOI) of approximately 50:1. Cell cultures were then incubated at 37 °C to allow for the adhesion and internalization of bacteria. After 2 h, cells were washed twice with 1 ml of DMEM, and unbound bacteria were discarded.

2.4. Flow cytometry-based assay (FCBA)

After the infection step, the cells used for the adhesion assay were trypsinized, washed twice in ice-cold phosphate buffer saline (PBS), and incubated in the dark with a 1:1 mixture of vancomycin and vancomycin-Bodipy FL® (VBFL) fluorochrome (Invitrogen) at a concentration of 0.8 μg/ml (100 μl/sample) for 15 min on ice (VBFL specifically binds the cell wall peptidoglycan of Gram-positive bacteria and does not penetrate intact cells).

After the infection step, the cells used for the invasion assay were incubated with 10 μg/ml of membrane-impermeable lysostaphin for 20 min to disrupt the extracellular bacteria. The intracellular bacteria were not disrupted. Then, the cells were washed, trypsinized and permeabilized on ice using 0.1% saponin in PBS for 20 min to allow for the cell penetration of VBFL. After a triple wash with ice-cold PBS, cells bearing intracellular bacteria were labeled with VBFL as described above. A subset of cells treated with lysostaphin without permeabilization was labeled with VBFL to serve as the negative control and to confirm the complete lysis of the extracellular bacteria.

After labeling, the cells for both adhesion and invasion assays were washed twice with PBS, fixed in 1% formol and stored at 4 °C prior to flow cytometry analysis.

The fixed samples were analyzed on a Canto II cytometer (Becton Dickinson) using the FL-1 channel to measure fluorescence intensity and the FSC/SSC stopping gate to exclude cellular debris and unbound bacteria (Fig. 1). The fluorescence intensity marker *M* was set to include less than 2% of the negative control (uninfected) cells. The total number of bacteria was estimated as the mean fluorescence intensity of cells in *M* multiplied by the proportion of cells in *M*. This measurement was expressed in arbitrary fluorescence units (AFUs) (Sinha et al., 1999). The data were represented as the mean ± standard deviation (SD) of four independent experiments performed in duplicate.

2.5. Gentamicin protection assay (GPA)

For the adhesion assay, infected cells were lysed using osmotic shock in pure sterile water with extensive pipetting immediately following the infection step. Viable adherent and internalized bacteria released after host cell lysis were enumerated by serial dilution and plate counting on agar plates.

For the internalization assay, cells were incubated after the infection step with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. The cells were then lysed, and viable bacteria were counted as described above. The data were represented as the mean ± standard deviation (SD) of four independent experiments performed in duplicate.

The coefficients of variation (CV%) of the results obtained using the FCBA and the GPA were calculated for comparative analysis.

2.6. Microscopic evaluation of extra- and intracellular bacteria

Two days before infection, MG-63 cells were seeded on glass coverslips at 50,000 cells/well in 24-well culture plates and incubated in culture medium at 37 °C in 5% CO_2. Then, cells were washed twice with DMEM and infected with *S. aureus* at a MOI of 10:1 for 2 h.

Then, glass coverslips for the adhesion assay were washed twice and cells were fixed with 4% paraformaldehyde in PBS for 30 min and incubated in the dark with 250 μl of a 1:1 mixture of vancomycin and vancomycin-Bodipy FL® (VBFL) fluorochrome (Invitrogen) at a 4 μg/ml concentration for 8 min. After a triple wash with ice-cold PBS, cells were permeabilized on ice using 0.05% Triton X100 for 15 min on ice. Cells were washed three times, and F-actin was stained using TRITC-conjugated rhodamine–phalloidin fluorescein (200 μl, 165 nM) (Sigma-Aldrich, St. Louis, MO) at RT.

Cells for the invasion assay were incubated with 10 μg/ml of lysostaphin for 20 min to disrupt the extracellular bacteria. Then, they were washed twice, fixed and permeabilized as described in Section 2.4. Then, F-actin and intracellular bacteria were labeled as described above.

Finally, the cells for both adhesion and invasion assays were washed three times with PBS, fixed and coverslips were dried. Photos were taken using a Leica DFC 420 C digital camera. Fluorescence signals of labeled specimens were serially recorded with appropriate excitation and emission filters, to avoid bleed-through. Images were digitally processed with Photoshop 6.0 (Adobe Systems, Mountain View, CA).

2.7. Statistical analysis

The differences in continuous variables were tested for statistical significance using Student's *t*-test without an equal variance assumption (Cochran–Cox method). The significance threshold was set at 0.05.

3. Results

3.1. Flow cytometry analysis

The adhesion to and the internalization by MG-63 cells of *S. aureus* strain 8325-4 and its isogenic mutant DU5883 (Δ*fnbA/B*) were compared by flow cytometry after selective VBFL-labeling of bacteria. The adhesion AFUs for strains 8325-4 and DU5883 were 4.07 ± 1.35 and 0.93 ± 0.27, respectively ($p<0.001$), whereas the invasion AFUs were 8.21 ± 2.4 and 0.2 ± 0.13, respectively ($p<0.001$). The background fluorescence controls (uninfected cells) for the adhesion and invasion assays were low (AFU = 0.07 ± 0.03 and 0.07 ± 0.01, respectively). This finding demonstrates the specificity of VBFL for bacterial peptidoglycan. The efficient disruption of extracellular bacteria by lysostaphin treatment was confirmed by the absence of fluorescence staining outside of control cells for both strains (AFU = 0.65 ± 0.25 and 0.09 ± 0.06 for strains 8325-4 and DU5883, respectively). To allow comparisons to the data obtained using the GPA, the results

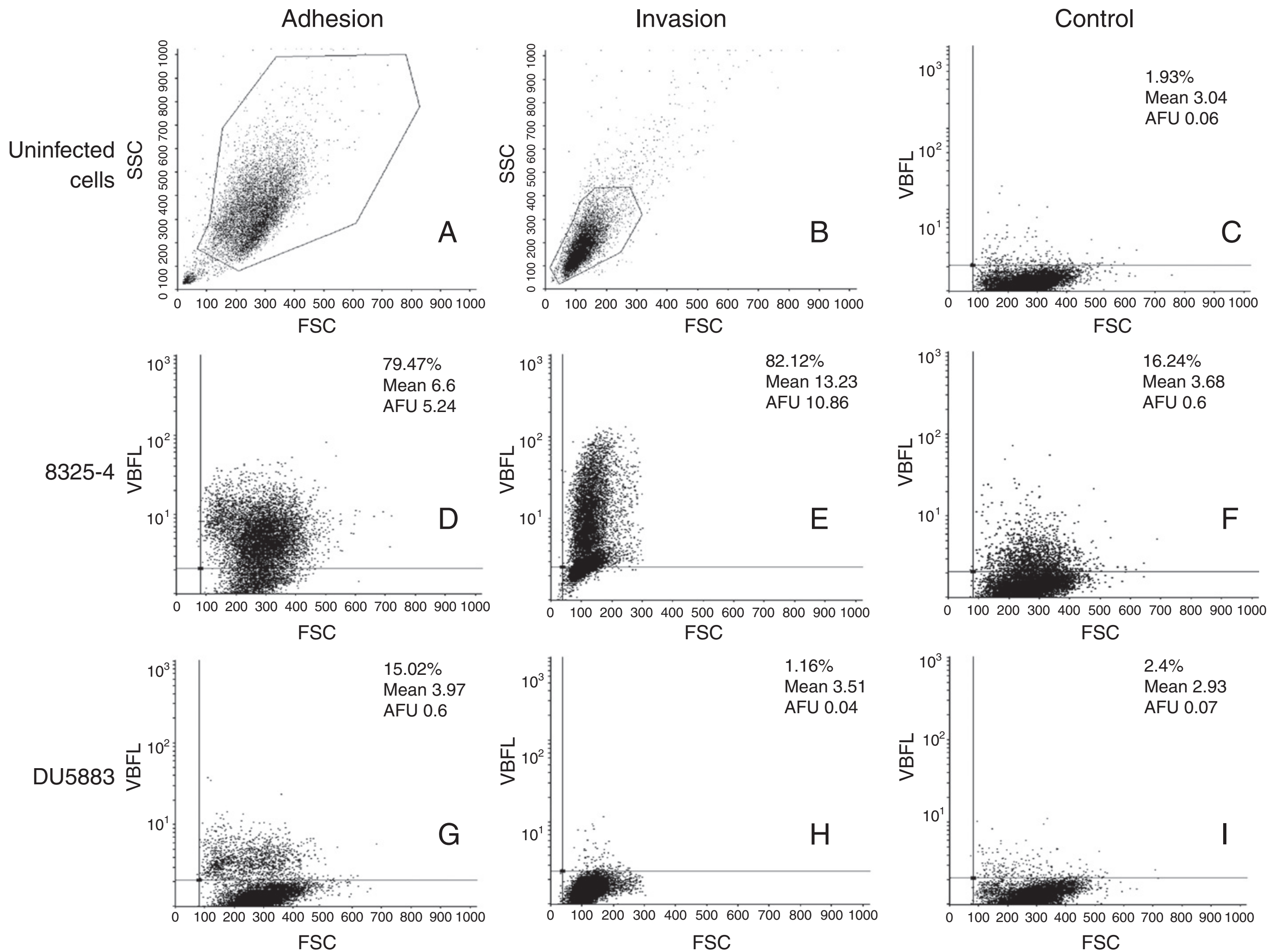

Fig. 1. Flow cytometry quantification of adhesion to and invasion of MG-63 cells by *S. aureus* strain 8325-4 and its invasion-defective isogenic mutant DU5883 (Δ*fnbA/B*). The results are presented as the original dot plots of a representative experiment. Flow cytometry analysis was performed using an FSC/SSC stopping gate with untreated, uninfected cells (A, adhesion assay) or uninfected cells permeabilized by saponin (B, invasion assay). The fluorescence intensity marker *M* was set to include <2% of uninfected cells (C). The values in each panel are the percentage of cells in *M*, the mean fluorescence of cells in *M*, and the arbitrary fluorescence unit (AFU) value that corresponds to the multiplication of the two previous parameters. For the adhesion assay, extracellular bacteria were labeled using the membrane-impermeable fluorochrome vancomycin-Bodipy FL (VBFL) (D, G). For the invasion assay, extracellular bacteria were disrupted by lysostaphin treatment, cells were permeabilized by saponin and intracellular bacteria were labeled with VBFL (E, H). The infected cells that were treated with lysostaphin without permeabilization and labeled with VBFL served as a negative control and confirmed the disruption of extracellular bacteria (F, I).

were also expressed as percentages of AFUs obtained for strain 8325-4 (Fig. 2).

3.2. Gentamicin protection assay

The results of the GPA were expressed as percentages of colony-forming units (CFUs) per well recovered for strain 8325-4 (Fig. 2). The adhesion level of strain DU5883 was 52.1 ± 20.4%, $p<0.01$, whereas the invasion level of the same strain was virtually abolished (0.4 ± 0.4%, $p<0.001$).

3.3. Microscopic evaluation of extra- and intracellular bacteria

Fixed samples were labeled with VBFL and examined microscopically to confirm results obtained with flow cytometry. After infection step, adherent staphylococci were extracellularly observed (arrow, Fig. 3). In agreement with flow cytometry, after lysostaphin incubation, extracellular staphylococci were disrupted, and no adherent bacteria were detectable. Finally, after lysostaphin incubation and permeabilization, the inspection of infected samples showed the presence of intracellular bacteria (arrowhead, Fig. 3).

4. Discussion

Robust and time-efficient methods for quantifying staphylococcal adhesion to and internalization by host cells are crucially needed in the research field of host–pathogen interactions. The flow cytometry-based assay presented here provides a new tool for simultaneously assessing the adhesion and invasion properties of unprocessed *S. aureus* in an *ex vivo* cell culture model. We used the fluorescent vancomycin derivative VBFL, which specifically binds to the cell wall of Gram-positive bacteria, to label membrane-bound and internalized *S. aureus* in less than 15 min.

Previous reports have established that either FnBP A or B is required for *S. aureus* host cell invasion (Sinha et al., 2000). Thus, the use of an isogenic couple of *S. aureus* strains (8325-4 and DU5883 [Δ*fnbA/B*]) allowed us to assess the specificity of the FCBA method for both adhesion and invasion. The differences in the adhesion and invasion levels of this isogenic couple were in agreement with

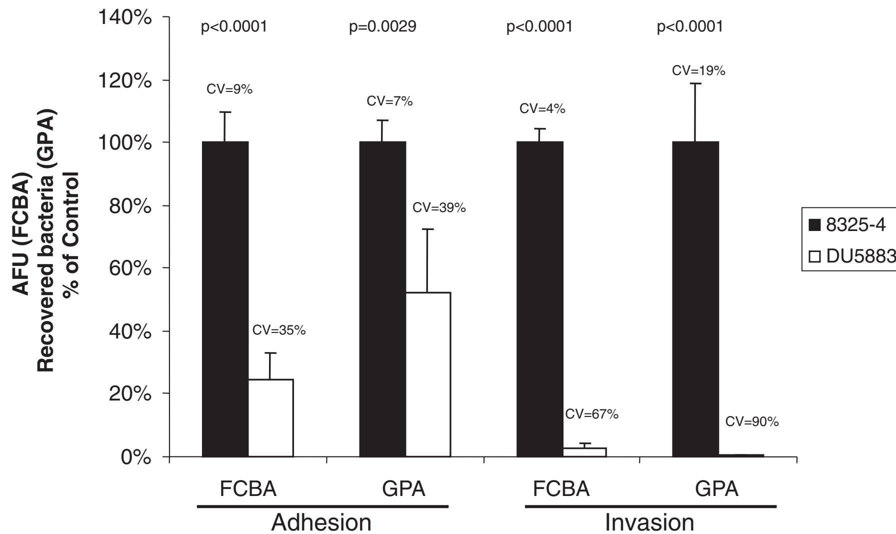

Fig. 2. Comparison of the adhesion and invasion levels of *S. aureus* strain 8325-4 and its invasion-defective isogenic mutant DU5883. Adhesion and invasion into MG-63 cells were quantified using either the flow cytometry-based assay (FCBA) or the gentamicin protection assay (GPA). Means ± SD of arbitrary fluorescence units (AFUs) measured using FCBA, and CFUs per well measured using GPA, were expressed as percentages of values obtained for strain 8325-4, derived from four independent experiments performed in duplicate. Coefficients of variation (CV%) were calculated to compare the reproducibility of the two methods.

previously published results and reached statistical significance (Sinha et al., 1999). The results obtained using the FCBA were also consistent with the results obtained using the widely used GPA. Two major observations emerged from the comparison between the FCBA and GPA results on a comparable ratio scale (Fig. 2). First, although the FCBA exhibited similar reproducibility to the GPA in terms of CV% for the adhesion assay, this reproducibility was appreciably improved with respect to the invasion assay. The FCBA offers an opportunity to be more discriminative when comparing *S. aureus* isolates or populations. Second, differences in adhesion between strain 8325-4 and its weakly adherent mutant were emphasized by comparing the FCBA to the GPA. A likely explanation for this is that weakly bound bacteria are more efficiently removed from the host cell surface during the centrifugation steps performed on the suspended cells used in the FCBA than during the cell monolayer washing procedure used in the GPA.

The updated flow cytometric approach described here presents several advantages. In contrast to methods using genetically modified or prelabeled bacteria, the FCBA uses post-adhesion and post-invasion processing of bacteria; thus, the FCBA avoids potential biases related to interference with bacterial viability and the physiological state during the infection process. Moreover, the FCBA allows for a rapid and simultaneous quantitative analysis of extracellular (adhesion) and intracellular (invasion) bacteria. A similar analysis is not possible when using genetically modified or prelabeled bacteria. Although the fluorescent signal of extracellular bacteria can be selectively attenuated by the use of a membrane-impermeable fluorescence quencher, such as trypan blue, there is no method described to date that allows specific quenching of intracellular bacteria. Moreover, the method presented here could potentially be applied to other eukaryotic cell models and Gram-positive bacteria on the basis of the high specific binding of vancomycin to bacterial peptidoglycan.

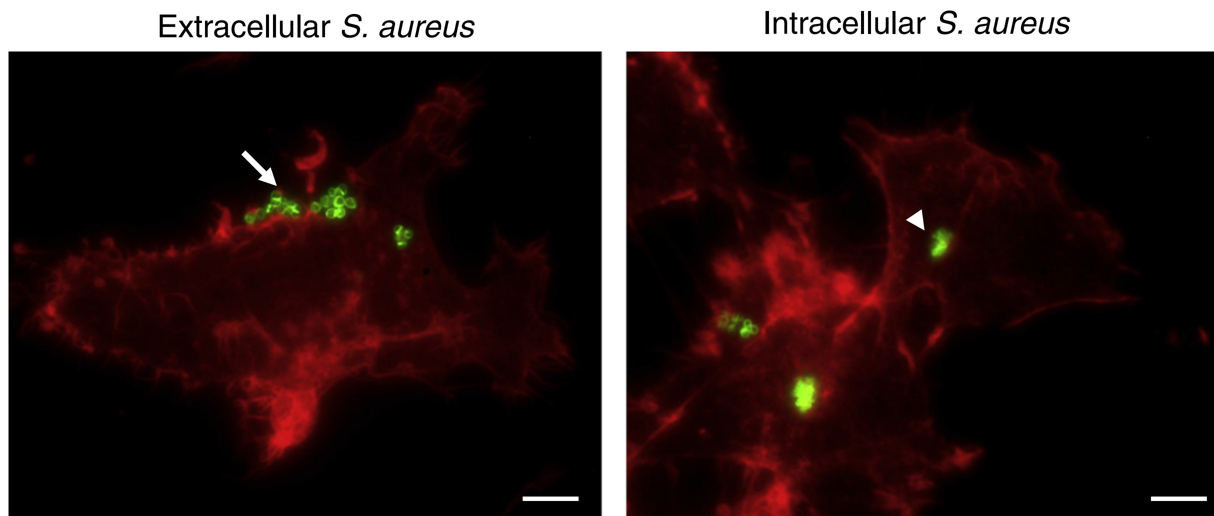

Fig. 3. Microscopic evaluation of extra- and intracellular *S. aureus*. MG-63 cells were infected with *S. aureus* reference strain 8325-4 for 2 h at a MOI of 10:1. For the adhesion assay, extracellular bacteria (arrow) were labeled using the membrane-impermeable fluorochrome vancomycin-Bodipy FL (VBFL). For the invasion assay, extracellular bacteria were disrupted by lysostaphin treatment, cells were permeabilized by Triton 0.05% and intracellular bacteria (arrowhead) were labeled with VBFL. The actin cytoskeleton of the cells was counterstained using phalloidin—red. Bars represent 10 μm.

Two limitations of the FCBA approach should be acknowledged. Bacteria used for the invasion assay must be completely disrupted (not only killed) by a membrane-impermeable compound that does not affect the host cell. We successfully used the endopeptidase lysostaphin to disrupt *S. aureus*. However, lysostaphin susceptibility has been shown to be modulated by various factors: i) culture media that can influence capsular polysaccharide production (Dassy et al., 1991; Stringfellow et al., 1991); ii) mutations affecting *femA*, a gene responsible for the addition of the second and third glycines to the pentaglycine cross-bridges in peptidoglycan (Kusuma et al., 2007); and iii) the acquisition of the endopeptidase resistant gene (*epr*) that results in lysostaphin resistance due to the substitution of serine for glycine in the pentaglycine cross-bridges (Dehart et al., 1995). All of these features might limit the panel of staphylococcal isolates that could be studied for invasion. The second limitation of the FCBA is that the AFU values obtained from the same isolate in the adhesion and invasion assays are independent because these values are obtained in separate wells. Thus, AFU values cannot be compared as CFU/well values are compared in the GPA.

5. Conclusions

This novel FCBA approach maintains the advantages of previously described cytometry-based invasion assays in terms of reproducibility and cost- and time-efficiency. This approach also introduces two additional features: the use of live and unprocessed bacteria during the infection step and the simultaneous quantification of host cell adhesion and invasion. Therefore, the FCBA method should be a useful addition to the evolving research field of *S. aureus*–host cell interactions.

Potential conflicts of interest

All the authors report no conflict of interest.

Acknowledgments

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References

Agerer, F., Waeckerle, S., Hauck, C.R., 2004. Microscopic quantification of bacterial invasion by a novel antibody-independent staining method. J. Microbiol. Methods 59, 23–32.

Ahmed, S., M.S., Williams, R.J., Henderson, B., Brock, J.H., Nair, S.P., 2001. *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. Infect. Immun. 60, 2872–2877.

Berendt, T., Byren, I., 2004. Bone and joint infection. Clin. Med. 4, 510–518.

Dassy, B., Stringfellow, W.T., Lieb, M., Fournier, J.M., 1991. Production of type 5 capsular polysaccharide by *Staphylococcus aureus* grown in a semi-synthetic medium. J. Gen. Microbiol. 137, 1155–1162.

Dehart, H.P., Heath, H.E., Heath, L.S., Leblanc, P.A., Sloan, G.L., 1995. The lysostaphin endopeptidase resistance gene (epr) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and *Staphylococcus aureus*. Appl. Environ. Microbiol. 61, 2811.

DeLoid, G.M., Sulahian, T.H., Imrich, A., Kobzik, L., 2009. Heterogeneity in macrophage phagocytosis of *Staphylococcus aureus* strains: high-throughput scanning cytometry-based analysis. PLoS One 4, e6209.

Ellington, J.K., Reilly, S.S., Ramp, W.K., Smeltzer, M.S., Kellam, J.F., Hudson, M.C., 1999. Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. Microb. Pathog. 26, 317–323.

Foster, T.J., Hook, M., 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol. 6, 484–488.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P., Lew, D., Foster, T., 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of fnb genes. Mol. Microbiol. 17, 1143–1152.

Jevon, M., Guo, C., Ma, B., Mordan, N., Nair, S.P., Harris, M., Henderson, B., Bentley, G., Meghji, S., 1999. Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. Infect. Immun. 67, 2677–2681.

Kusuma, C., Jadanova, A., Chanturiya, T., Kokai-Kun, J.F., 2007. Lysostaphin-resistant variants of *Staphylococcus aureus* demonstrate reduced fitness *in vitro* and *in vivo*. Antimicrob. Agents. Chemother. 51, 475–482.

Pils, S., Schmitter, T., Neske, F., Hauck, C.R., 2006. Quantification of bacterial invasion into adherent cells by flow cytometry. J. Microbiol. Methods 65, 301–310.

Sinha, B., Francois, P., Nube, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., Krause, K.H., 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasion via fibronectin bridging to integrin α5β1. Cell. Microbiol. 1, 101–107.

Sinha, B., Francois, P., Que, Y.A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K.H., Peters, G., Herrmann, M., 2000. Heterologously expressed *Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells. Infect. Immun. 68, 6871–6878.

Stevens, Q.E., Seibly, J.M., Chen, Y.H., Dickerman, R.D., Noel, J., Kattner, K.A., 2007. Reactivation of dormant lumbar methicillin-resistant *Staphylococcus aureus* osteomyelitis after 12 years. J. Clin. Neurosci. 14, 585–589.

Stringfellow, W.T., Dassy, B., Lieb, M., Fournier, J.M., 1991. *Staphylococcus aureus* growth and type 5 capsular polysaccharide production in synthetic media. Appl. Environ. Microbiol. 57, 618–621.

Wright, K.M., Friedland, J.S., 2004. Regulation of chemokine gene expression and secretion in *Staphylococcus aureus*-infected osteoblasts. Microbes. Infect. 6, 844–852.

III. DEUXIÈME PUBLICATION : HYPERVIRULENT *STAPHYLOCOCCUS AUREUS* INDUCES DEATH OF INFECTED OSTEOBLASTS BY AN INTRACELLULAR PHENOL-SOLUBLE MODULIN - DEPENDENT MECHANISM

Jean-Philippe Rasigade, Sophie Trouillet, Tristan Ferry, Binh An Diep, Anaïs Sapin, Yannick Lhoste, Jérémy Ranfaing, Cédric Badiou, Yvonne Bénito, Michèle Bes, Sylvestre Tigaud, Gérard Lina, Jérôme Etienne, François Vandenesch, Frédéric Laurent.

Soumis pour publication à *PloS Pathogens*

L'émergence et la diffusion rapide des CA-MRSA, associées à leur virulence particulière, a modifié la prise en charge et le pronostic des IOA dans les pays à forte prévalence comme les Etats-Unis. Par comparaison avec leurs homologues hospitaliers, les HA-MRSA, les CA-MRSA sont responsables d'ostéomyélites d'évolution plus aiguë, de pronostic plus sévère, nécessitant un recours quasi-sytématique à la chirurgie et une admission fréquente en réanimation ou en soins intensifs. L'état actuel des connaissances indique que la PVL est impliquée dans la physiopathologie des IOA à CA-MRSA ; cette toxine joue un rôle indirect dans la destruction osseuse en recrutant des leucocytes au site de l'infection et en provoquant leur activation et leur lyse, libérant in situ leur contenu granulaire et lysosomal toxique à l'origine d'une nécrose tissulaire. Cependant, les interactions directes ostéoblastes- CA-MRSA n'ont jamais fait l'objet d'études spécifiques jusqu'à ce jour. La nature multi-factorielle de la virulence des CA-MRSA ayant été clairement documentée, il nous est apparu pertinent de rechercher, au-delà de l'action indirecte

de la PVL, l’existence de mécanismes de virulence qui impliqueraient une destruction ciblée des ostéoblastes.

Notre hypothèse de travail était la suivante : les CA-MRSA sont capables d’envahir et détruire les ostéoblastes de façon plus efficace que les HA-MRSA. Sous cette hypothèse, les CA-MRSA présenteront une cytotoxicité supérieure aux HA-MRSA dans un modèle d’infection intracellulaire d’ostéoblastes humains. Nous avons validé cette hypothèse sur un panel de 35 souches de CA-MRSA et HA-MRSA incluant les principales lignées circulant dans le monde, et montré que la cytotoxicité des CA-MRSA vis-à-vis des ostéoblastes n’est pas l’apanage d’un seul clone mais bien un facteur commun aux différents clones de CA-MRSA.

Nous avons alors cherché à déterminer s’il existait un facteur de virulence spécifiquement associé à la cytotoxicité. Nous avons centré nos investigations sur les trois principaux déterminants de virulence des CA-MRSA, la PVL, la surexpression d’α-toxine et la surexpression des PSMs. La comparaison des phénotypes cytotoxiques de couples de souches isogéniques pour la PVL a permis d’éliminer un rôle de cette dernière dans la mort des ostéoblastes. Le niveau d’expression d’α-toxine, quant à lui, n’était pas corrélé à la cytotoxicité. De façon surprenante, l’inactivation du gène codant l’α-toxine chez deux souches de CA-MRSA différentes était même associée à une augmentation du phénotype cytotoxique ; ce résultat est discuté dans la troisième partie de cette thèse. Enfin, nous avons montré que l’inactivation des gènes codant les PSM de type α était associée à une diminution de cytotoxicité, démontrant un rôle de ces derniers dans la virulence des CA-MRSA vis-à-vis des ostéoblastes.

Parallèlement, nous avons investigué la cytotoxicité de mutants isogéniques délétés sur les principaux régulateurs de l’expression des toxines, *agr*, *sar*A et *sae*RS, pour déterminer quelles voies de régulation étaient associées à la cytotoxicité. L’expression d’un phénotype cytotoxique était dépendante du caractère fonctionnel d’*agr* et *sar*A, mais pas de celui de *sae*RS. Il est intéressant de noter qu’*agr* et *sar*A contrôlent étroitement l’expression des PSMs, alors que *sae*RS ne l’influence pas. Ainsi, la nature des voies de régulation associées à la cytotoxicité est en faveur du rôle des PSM.

Enfin, nous avons employé une approche de génotypage pour rechercher d'éventuelles associations entre la cytotoxicité et la présence de gènes de virulence. Chez l'ensemble des 35 souches de MRSA étudiées, 126 gènes d'intérêt codant des toxines, des adhésines, des régulateurs ou des déterminants de résistance, ont été recherchés par puce à ADN. La présence d'associations statistiques entre chaque gène et la cytotoxicité a été recherchée par régression linéaire multivariée. Cette analyse n'a pas permis d'identifier d'association entre les gènes étudiés et le phénotype cytotoxique. Nous considérons que ce résultat est compatible avec le rôle des PSM dans la cytotoxicité : ces toxines font partie du génome cœur de *S. aureus*, par conséquent leur rôle dans la virulence spécifique de chaque souche est liée à leur niveau d'expression, qui ne peut être investigué par une méthode génomique basée sur la présence/absence des gènes.

L'ensemble de ces résultats améliore notre connaissance de la physiopathologie des IOA à CA-MRSA à plusieurs titres. Premièrement, le fait que ces pathogènes soient capable d'induire la mort des ostéoblastes infectés de façon plus efficace que les HA-MRSA dévoile un nouveau mécanisme de virulence, indépendant de la PVL et de l'α-toxine. Deuxièmement, ces résultats démontrent que contrairement au modèle établi, le rôle physiopathologique de l'internalisation de *S. aureus* par les ostéoblastes ne procède pas toujours d'un échappement à l'action des antibiotiques et du système immunitaire avec passage à l'état quiescent et sanctuarisation de *S. aureus* (à l'origine de rechutes) comme cela semble le cas chez les HA-MRSA, mais peut au contraire, chez les CA-MRSA, participer d'une stratégie agressive aboutissant à la mort de la cellule envahie. Enfin, nous apportons la première démonstration d'une toxine à action intracellulaire chez *S. aureus* : les PSM, dont le rôle comme toxine extracellulaire a récemment été remis en question (Surewaard *et al.*, 2012), confèrent aux CA-MRSA la capacité de tuer leur cellule hôte après invasion de celle-ci. Il est notable que l'action des PSM semble indépendante de leur fixation à un récepteur spécifique (Wang *et al.*, 2007). Ainsi, leur surexpression par les CA-MRSA est susceptible d'induire la mort de toute cellule capable de phagocytose. Les implications de ces résultats seront discutées dans la troisième partie de cette thèse.

Contribution personnelle au travail : apport de la problématique, conception du projet, mise au point du modèle expérimental, réalisation des expériences ex vivo et encadrement d’étudiants ayant réalisé des expériences ex vivo (A. Sapin, Y. Lhoste et J. Ranfaing), analyse et interprétation des données, rédaction du manuscrit.

Contributions majeures non réalisées par l’auteur : les constructions génétiques ont été réalisées par Y. Benito et B.A. Diep ; les analyses de génotypage ont été réalisées par l’équipe technique du CNR des Staphylocoques et coordonnées par M. Bes ; la quantification d’α-toxine par méthode ELISA a été réalisée par C. Badiou.

Hypervirulent *Staphylococcus aureus* Induces Death of Infected Osteoblasts by an Intracellular Phenol-Soluble Modulin-Dependent Mechanism

Jean-Philippe Rasigade[1,2,3]*, Sophie Trouillet[1], Tristan Ferry[1], Binh An Diep[4], Anaïs Sapin[3], Yannick Lhoste[3], Jeremy Ranfaing[1], Cédric Badiou[1], Yvonne Benito[2], Michèle Bes[2], Sylvestre Tigaud[3], Gerard Lina[1,2], Jerome Etienne[1,2], François Vandenesch[1,2], Frederic Laurent[1,2,3]

1 INSERM U851, University of Lyon, Lyon, France, **2** National Reference Center for Staphylococci, Hospices Civils de Lyon, Lyon, France, **3** Department of Clinical Microbiology, Northern Hospital Group, Hospices Civils de Lyon, Lyon, France, **4** Division of Infectious Diseases, Department of Medicine, University of California, San Francisco, CA

* Corresponding author:

Jean-Philippe Rasigade

INSERM U851, Faculté de Médecine Lyon-Est

7 rue Guillaume Paradin

69372 LYON CEDEX 8 – France

Phone: + 33 (0) 478 77 86 57; Fax: + 33 (0) 478 77 86 58

Email: jean-philippe.rasigade@chu-lyon.fr

Running title: CA-MRSA PSMs Induce Death in Osteoblasts

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21 **Abstract**

22 Epidemic community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is
23 associated with more severe and acute forms of osteomyelitis than healthcare-associated
24 (HA-) MRSA. Although *S. aureus* is now recognized as a facultative intracellular pathogen,
25 the contribution of osteoblast invasion by CA-MRSA to the pathogenesis of osteomyelitis is
26 unknown. Using an ex vivo model of intracellular infection of human osteoblasts, we
27 demonstrated that CA-MRSA strains of diverse lineages share an enhanced ability to kill
28 infected osteoblasts compared to HA-MRSA. Cytotoxicity comparisons of CA-MRSA isogenic
29 deletion mutants revealed that phenol-soluble modulins (PSMs), a class of membrane-
30 damaging exoproteins that are expressed at higher levels in CA-MRSA than in HA-MRSA,
31 are involved in this osteoblast killing, whereas other major CA-MRSA virulence determinants,
32 the Panton-Valentine leukocidin and alpha-toxin, are not involved. Similarly, functional *agr*
33 and *sar*A regulators, which control the expression of PSMs and alpha-toxin, were required for
34 the expression of the intracellular cytotoxic phenotype by CA-MRSA, whereas the *sae*RS
35 regulator, which controls the expression of alpha-toxin but not PSMs, had no impact on
36 cytotoxicity. These findings provide new insights into the pathogenesis of severe CA-MRSA
37 osteomyelitis and elucidate a novel virulence strategy of CA-MRSA based on the invasion
38 and PSM-related killing of osteoblasts.

39

40 **Introduction**

41 *Staphylococcus aureus* is the leading cause of osteomyelitis, which is defined as an
42 infection of the bone [1]. This versatile pathogen has evolved a remarkable ability to resist
43 antibiotics such as methicillin and other beta-lactams, complicating the management of
44 osteomyelitis [2]. Until the 1990s, methicillin resistance was recognized as a specific trait of
45 healthcare-associated *S. aureus* (HA-MRSA), which was first described in the early 1960s
46 [3]. The incidence of community-acquired (CA-) MRSA infections has since dramatically
47 increased in several countries [4], and this pandemic has altered the clinical landscape of
48 osteomyelitis, particularly in the pediatric setting [5, 6]. In the United States, CA-MRSA
49 infections are more frequent than their methicillin-susceptible counterparts [7-10], and the
50 dissemination of these strains has been coincident with an increase in both the incidence
51 and the severity of osteomyelitis [5, 9-12]. Children with osteomyelitis caused by CA-MRSA,
52 compared to other *S. aureus* lineages, exhibit greater systemic inflammatory responses [13],
53 experience longer durations of fever and longer hospital stays [5, 10], and more frequently
54 require surgical procedures [5]. Case series also suggested that these patients often require
55 admission to the intensive care unit [6, 9]. Notably, CA-MRSA infections have added to,
56 rather than replaced, infections caused by other microorganisms, including methicillin-
57 susceptible *S. aureus* (MSSA).

58 Investigations of the basis of CA-MRSA virulence are crucial for understanding its
59 pathogenesis and the development of novel therapeutics against these recently emerged
60 pathogens. Data from in vitro and animal models have shown that the virulence potential of
61 CA-MRSA is multifactorial. This virulence potential has evolved via the acquisition of the *pvl*
62 genes encoding the Panton-Valentine leukocidin (PVL) and through the increased
63 expression of core genome-encoded toxins, mainly alpha-toxin and phenol-soluble modulins
64 (PSMs) [8]. These pore-forming toxins induce apoptosis and lysis in different cell types. PVL

65 and PSMs target immune effector cells such as neutrophils [8], while alpha-toxin targets a
66 much wider spectrum of cells, including erythrocytes, alveolar epithelial cells [14], endothelial
67 cells [15], lymphocytes, and monocytes [16]. Experimental investigations of CA-MRSA
68 virulence have mainly focused on models of skin and soft tissue infections or pneumonia
69 because these diseases are the most frequent or the most severe, respectively, in the
70 spectrum of CA-MRSA infections [17]. As a consequence, few experimental data are
71 available regarding the pathogenesis of CA-MRSA osteomyelitis. Although PVL has been
72 shown to contribute to the severity of infection in a rabbit model of osteomyelitis [18], the
73 roles of other CA-MRSA-specific virulence mechanisms are unknown. Direct interactions of
74 *S. aureus* with osteoblasts are crucial in the pathogenesis of osteomyelitis [19, 20]. The
75 ability of *S. aureus* to invade and gain access to the cytoplasm of so-called non-professional
76 phagocytes such as osteoblasts has gained increased attention [19-22] and is now regarded
77 as a key factor in therapy-refractive infections [23, 24].

78 The primary objective of this work was to compare the ability of CA-MRSA and HA-
79 MRSA strains to invade and damage human osteoblasts in an ex vivo model. To achieve
80 adequate representation of MRSA strains circulating worldwide, 35 strains of the major CA-
81 MRSA and HA-MRSA lineages were investigated. Our secondary objective was to determine
82 if specific virulence determinants were associated with either osteoblast invasion or killing by
83 MRSA. The roles of PVL, alpha-toxin, and PSM production and of the regulators *agr*, *sar*A,
84 and *sae*RS in the virulence of MRSA during intracellular infection were examined. To extend
85 the study beyond these specific factors, the genomic contents of the CA-MRSA and HA-
86 MRSA strains were compared using DNA microarrays, and genetic associations with
87 cytotoxicity and osteoblast invasion were investigated.

88 **Results**

89 **Intracellular CA-MRSA causes higher osteoblast damage than HA-MRSA**

90 We examined the cytotoxicity induced in human osteoblasts by 35 genetically diverse
91 clinical strains of MRSA selected from the collection of the French National Reference Center
92 for Staphylococci. These strains belonged to 3 major lineages of *pvl*$^+$ CA-MRSA, namely
93 sequence type (ST)8, pulsotype USA300, staphylococcal chromosomal cassette *mec*
94 (SCC*mec*) IV (ST8-USA300-IV clone), the ST80-IV European clone, and the ST30-
95 USA1100-IV Southwest Pacific clone [25], and to 4 major lineages of HA-MRSA, namely the
96 ST239-III Brazilian clone, the ST228-I Southern Germany clone, the ST8-EMRSA2-IV Lyon
97 clone, and the ST22-EMRSA15-IV Barnim clone (n = 5 strains each) [26, 27].

98 The infection protocol was comprised of a 2 h co-culture step of MRSA and MG-63
99 osteoblastic cells in antibiotic-free medium with a bacteria-host cell ratio of 100, followed by a
100 selection step in medium containing gentamicin and lysostaphin to kill non-internalized
101 bacteria. After 24 h of incubation, the *S. aureus*-induced cytotoxicity was estimated by a
102 lactate dehydrogenase (LDH) assay. The results were reported as the mean and 95% CI of
103 the n-fold change in LDH compared to cells infected with the *S. aureus* reference strain
104 8325-4 (control); each strain was tested in duplicate.

105 A significant difference was observed in the capacity of CA-MRSA and HA-MRSA to
106 induce osteoblast damage after 24 h. The relative LDH release by CA-MRSA-infected cells
107 was 1.7-fold higher than that by HA-MRSA-infected cells (1.67 [1.53-1.81] vs. 0.99 [0.93-
108 1.04], respectively; $P < 0.0001$, Welch's *t*-test; Figure 1A and Table S1). ANOVA followed by
109 Tukey's HSD post-hoc test was used to determine if the cytotoxicity was also dependent on
110 the lineage of the strains. Pairwise comparisons showed that (i) any of the 3 CA-MRSA
111 lineages induced significantly higher LDH release than any of the 4 HA-MRSA lineages (P <

112 0.01 for all differences) and (ii) no significant difference in LDH release was observed
113 between the lineages within the CA-MRSA or HA-MRSA groups.

114 **The intracellular bacterial load is higher in HA-MRSA- than CA-MRSA-infected**
115 **osteoblasts**

116 The viable intracellular bacterial loads (VIBL) within MRSA-infected osteoblasts were
117 determined using the infection assay described above, followed by the osmotic lysis of
118 infected cells at 24 h post-infection to release the bacteria, which were enumerated by plate
119 counting. The results were expressed as the mean and 95% CI of the n-fold change in the
120 VIBL compared to cells infected with the *S. aureus* strain 8325-4 (control) and were derived
121 from the same experiments as those used to quantify cytotoxicity.

122 The relative VIBL was 3.5-fold higher in HA-MRSA-infected osteoblasts than in CA-
123 MRSA-infected osteoblasts (2.50 [2.15-2.84] vs. 0.72 [0.54-0.91], respectively; $P < 0.0001$;
124 Fig 1B and Table S1). The differences between the lineages were analyzed using the same
125 ANOVA procedure as described above. Pairwise comparisons showed that (i) the relative
126 VIBL was significantly higher among the 4 HA-MRSA lineages than the 3 CA-MRSA lineages
127 ($P < 0.05$ for all differences, Tukey's HSD test) and (ii) no significant difference in the relative
128 VIBL was observed between the lineages within the CA-MRSA or HA-MRSA groups.

129 **The lower intracellular bacterial load of CA-MRSA is not explained by host cell killing**

130 Following the observation that CA-MRSA induced both higher cytotoxicity and lower
131 VIBL than did HA-MRSA, we tested the hypothesis that cytotoxicity was negatively correlated
132 with VIBL. Because bacteria that kill their host cells are released into the extracellular space
133 and excluded from the intracellular bacterial pool, the higher cytotoxicity of a given strain
134 could directly yield a lower VIBL. We thus searched for an association between cytotoxicity
135 and VIBL with and without controlling for the CA-MRSA or HA-MRSA status of the strain.

136 Figure 1C shows a plot of relative LDH release against VIBL. The VIBL was significantly
137 associated with cytotoxicity levels upon simple regression analysis ($P < 0.0001$, F-test).
138 However, multiple linear regression controlling for CA-MRSA or HA-MRSA status
139 demonstrated that there was no independent association between VIBL and cytotoxicity ($P =$
140 0.6).

141 To further explore the relationships between bacterial invasion, intracellular
142 persistence, and the CA-MRSA or HA-MRSA status of the strains, kinetics experiments were
143 performed. In these experiments, the number of viable osteoblasts was quantified
144 microscopically using Trypan blue exclusion, and the numbers of viable intracellular bacteria
145 were quantified by plate counting. These experiments allowed us to estimate the number of
146 viable bacteria per viable osteoblast at different time points. The first time point was 3 h after
147 the beginning of the infection step to reflect the efficiency of the invasion process.
148 Subsequent time points were taken at 24 and 48 h after infection to investigate the clearance
149 of intracellular bacteria with respect to the initial VIBL. Two strains (the ST80-IV CA-MRSA
150 strain HT20020209 and the ST8-EMRSA2-IV HA-MRSA strain HT20040117) were randomly
151 selected from the 35 MRSA strains and included in these experiments (see arrows in Figure
152 1C). The results are reported as the means and 95% CI derived from three independent
153 experiments in triplicate. At 3 h post-infection, the osteoblasts harbored an average of 0.77
154 [0.52-1.03] ST80-IV cells and 3.59 [2.30-4.89] ST8-EMRSA2-IV cells, which corresponded to
155 approximate intracellular passages of 1% and 4%, respectively, of the bacterial inoculum set
156 at 100 bacteria per osteoblast (Figure 2A). These figures remained stable from 3 to 24 h
157 post-infection, at which time the bacteria per osteoblast ratios were 0.86 [0.44-1.27] and 5.78
158 [4.13-7.44] for the ST80-IV and ST8-EMRSA2-IV strains, respectively. Significant bacterial
159 clearance occurred between 24 and 48 h, at which time the ratios fell to 0.02 [0.01-0.03] for
160 the ST80-IV strain and 0.55 [0.06-1.03] for the ST8-EMRSA2-IV strain, corresponding to
161 46.3- and 10.6-fold reductions, respectively, in the bacterial load. Comparisons of the ST8-

162 EMRSA2-IV and ST80-IV strains revealed that the bacteria per osteoblast ratios after 3, 24,
163 and 48 h of incubation were 4.6-, 6.8-, and 29.5-fold higher, respectively, for the HA-MRSA
164 strain than for the CA-MRSA strain ($P < 0.05$ for all differences, Welch's *t*-test). Collectively,
165 these findings indicated that the invasion process itself and the ability to survive
166 intracellularly after invasion were less efficient in the CA-MRSA strain HT20020209 than in
167 the HA-MRSA strain. Moreover, these experiments confirmed that the difference in the
168 amounts of intracellular bacteria between CA-MRSA and HA-MRSA was independent of the
169 host cell death caused by CA-MRSA.

170 Additional experiments to investigate osteoblast infection were conducted as
171 described above using the same two strains, HT20020209 and HT20040117, to estimate the
172 mortality of infected osteoblasts. At 24 h post-infection, LDH release into the supernatant of
173 infected cells was compared to that of uninfected cells that were either left intact (lower
174 control) or fully lysed by osmotic shock (higher control). The percent mortality was calculated
175 as follows: (LDH infected cells - LDH lower control) / (LDH higher control - LDH lower
176 control). The results were reported as the means and 95% CI derived from three
177 independent experiments in triplicate. The percent mortality in osteoblasts infected with the
178 CA-MRSA strain HT20020209 and the HA-MRSA strain HT20040117 were 51.8% [46.6-
179 56.9] and 21.0% [16.6-25.5], respectively ($P < 0.0001$, Welch's *t*-test; Figure 2B). These
180 results, together with those of the infection kinetics experiments, confirmed the potent
181 cytotoxic activity of intracellular CA-MRSA by showing that an average intracellular load of
182 one bacterium per host cell resulted in the death of half of the host cell population by 24 h.

183 **The production of PSMs, but not PVL or alpha-toxin, is associated with osteoblast**
184 **damage**

185 The presence of PVL, along with the overexpression of alpha-toxin and PSMs relative
186 to other *S. aureus* lineages, is considered a major virulence trait of CA-MRSA [17, 28, 29].

187 Because these toxins induce cell death through various mechanisms, we investigated their
188 influence on the virulence phenotype observed in our model, as well as the influence of the
189 regulators *agr*, *sar*A, and *sae*RS, which control the expression of these toxins [29-32].

190 Although PVL specifically targets immune cells, this toxin has been shown to bind
191 mitochondria and to cause Bax-independent apoptosis through the mitochondrial pathway
192 [33]. Hence, direct delivery of PVL by intracellular CA-MRSA in the cytoplasm of infected
193 osteoblasts may allow the toxin to gain access to the mitochondria without the need for
194 immune cell type-specific binding to the plasma membrane. PVL is found in most CA-MRSA
195 but not HA-MRSA strains and is expressed at toxic levels as long as the corresponding
196 genes are present in the genome [34, 35]. Therefore, we used a loss-of-function approach to
197 examine the influence of PVL on cytotoxicity by using isogenic $pvl^{+/-}$ strains belonging to the
198 three CA-MRSA lineages investigated in the previous experiments. With respect to ST8-
199 USA300-IV, strains LAC and SF8300, as well as their Δ*pvl* derivatives LACΔ*pvl* and
200 SF8300Δ*pvl*, have been described previously [36, 37]. The following mutants were
201 constructed by allelic replacement: the LUG1800 Δ*pvl* mutant of the ST80-IV strain
202 HT20020209, and the BD0448 Δ*pvl* mutant of the ST30-USA1100-IV strain BD0428. The
203 cytotoxicity toward osteoblasts was assessed after 24 h of infection using the same
204 procedure as described above. The results of three experiments performed in triplicate are
205 presented in Figure 3A. No significant differences in cytotoxicity were observed between the
206 wild-type and Δ*pvl* strains in the three lineages investigated ($P > 0.05$ for all comparisons,
207 Welch's *t*-test), thus eliminating a potential role for PVL in the increased cytotoxicity of CA-
208 MRSA toward osteoblasts.

209 The *hla* gene encoding alpha-toxin belongs to the core genome of *S. aureus*, and the
210 expression level of this toxin has been shown to affect strain-specific virulence [38]. We thus
211 combined quantitative and loss-of-function approaches to investigate the influence of alpha-

212 toxin on cytotoxicity. The in vitro production of alpha-toxin by MRSA strains and by the
213 *S. aureus* strain 8325-4 was quantified in duplicate using a sandwich ELISA and reported as
214 ng/mL. Because the data were not normally distributed upon visual inspection, we used non-
215 parametric tests for the statistical analysis and reported the medians and interquartile ranges
216 (IQR) instead of means and the 95% CI. Alpha-toxin production tended to be higher in CA-
217 MRSA than in HA-MRSA strains, but this difference did not reach statistical significance
218 (median and IQR, 5153 ng/mL [1790-7683] vs. 2310 ng/mL [36-4326], respectively; P =
219 0.074, two-tailed Mann-Whitney U-test; Figure 4A and Table S1). Among the 35 MRSA
220 strains investigated, 7 strains produced low amounts of alpha-toxin (< 50 ng/mL), including
221 the 5 ST228-I HA-MRSA strains (100%), 1 ST8-EMRSA2-IV HA-MRSA strain (20%), and
222 unexpectedly, 1 ST8-USA300-IV CA-MRSA strain (20%). We plotted the relative cytotoxicity
223 of the MRSA strains against the alpha-toxin activity (Figure 4B) and searched for an
224 association between these factors using a non-parametric correlation analysis. A moderate
225 rank correlation was found (Spearman's coefficient = 0.31) that did not reach statistical
226 significance (P = 0.069). No association was found by multiple linear regression analysis
227 controlling for the CA-MRSA or HA-MRSA status (P = 0.75, F-test). Notably, the 8325-4
228 control strain, which had the highest alpha-toxin production (28.8 µg/mL) due to a previously
229 described chromosomal defect [39], was less cytotoxic toward osteoblasts than any of the
230 CA-MRSA strains, including the alpha-toxin-deficient USA300 strain. In addition, the
231 osteoblast cytotoxicity induced by the CA-MRSA strains LAC and SF8300, as well as their
232 respective Δ*hla* isogenic derivatives LACΔ*hla* (kindly provided by F. DeLeo [28]) and
233 SF8300Δ*hla*, were compared (Figure 3C). Inactivation of the *hla* gene in both the LAC and
234 SF8300 strains was not associated with decreased cytotoxicity; conversely and
235 unexpectedly, the Δ*hla* strains exhibited a stronger cytotoxic phenotype than their parental
236 strains (P < 0.001 for both differences). Collectively, these findings indicate that alpha-toxin
237 does not contribute to the increased cytotoxicity of CA-MRSA toward osteoblasts in our

238 model and suggest that alpha-toxin expression may actually impair the osteoblast damage
239 caused by CA-MRSA.

240 PSMs are core genome-encoded amphipathic peptides that have been associated
241 with CA-MRSA virulence in animal models [29, 40, 41] and are able to recruit, activate, and
242 lyse neutrophils [40, 42]. The cytotoxic phenotypes of the previously described CA-MRSA
243 strain SF8300 and of its isogenic derivative SF8300Δ*psm*α1-4, which lacks alpha-type
244 PSMs, were compared [43] (Figure 3B). The inactivation of the alpha-type PSMs induced a
245 significant decrease in osteoblast damage after 24 h of incubation, indicating that the
246 expression of alpha-type PSMs by CA-MRSA is associated with a cytotoxic phenotype.

247 Toxin expression in *S. aureus* is tightly controlled by a regulatory network involving
248 several regulators, including *agr*, *sar*A, and *sae*RS [38]. The increased toxin expression and
249 virulence of CA-MRSA strains has been attributed to the increased expression of these
250 systems [32, 38]. We thus investigated the respective contributions of each of these 3
251 regulators to cytotoxicity by constructing isogenic derivatives of strain SF8300 that lack *agr*A,
252 *sar*A, or *sae*RS. Both the SF8300Δ*agr* and SF8300Δ*sar*A strains but not the SF8300Δ*sae*RS
253 strain induced less damage in infected osteoblasts than the wild-type SF8300 strain (Figure
254 3D). These results indicate that the virulence determinants responsible for osteoblast death
255 after invasion are under the control of *agr* and *sar* but not *sae*RS.

256 **DNA microarray analysis of MRSA strains**

257 To determine if the differences in the cytotoxicity and VIBL of the CA-MRSA and HA-
258 MRSA strains were related to specific virulence factors other than PVL, alpha-toxin, or PSMs,
259 the 35 MRSA strains were screened for the presence of 126 genes by the use of StaphyType
260 DNA microarrays (Alere Technologies GmbH, Jena, Germany) (Table S2) [44]. Associations
261 between cytotoxicity levels, intracellular persistence, the CA- or HA-MRSA status of the

262 strain, and the presence of each gene were screened using linear regression for numerical
263 response variables and the two-tailed Fisher's exact test for categorical response variables.
264 In accordance with the exploratory nature of this analysis, *P*-values were not corrected for
265 multiple testing.

266 The distributions of 18 genes were significantly associated with the CA-MRSA or HA-
267 MRSA status of the strains (Table 1). Most of these genes were associated with specific
268 lineages (e.g., *ent*A was associated with the ST239-III, ST228-II, and ST8-EMRSA2-IV
269 lineages), reflecting the close genetic relationships between strains of a given lineage. With
270 the exception of the *pvl* locus, none of the genes investigated showed a clear dichotomous
271 distribution between the CA-MRSA and HA-MRSA strains.

272 The genes whose presence was significantly associated with cytotoxicity by simple
273 linear regression analysis included (in order of increasing *P*-values) the following: the *pvl*
274 locus, *ent*A (enterotoxin A), *erm*A (erythromycin resistance determinant), *qac*A (quaternary
275 ammonium cation resistance determinant), the *mer* operon (mercury resistance determinant),
276 *et*D (exfoliative toxin gene D), *edin*B (epidermal cell differentiation inhibitor B), *aac*A-*aph*D
277 and *aad*D (aminoglycoside resistance determinants), and *fnb*B (fibronectin-binding protein B)
278 ($P < 0.05$, *F*-test). Interestingly, all of these genes were among those differentially distributed
279 between the CA-MRSA and HA-MRSA strains (Table 1). Multiple regression analysis
280 controlling for the CA-MRSA or HA-MRSA status of the strain failed to demonstrate an
281 independent association between cytotoxicity and any of the 126 genes investigated.

282 The genes associated with the relative VIBL by simple linear regression analysis were
283 (in order of increasing *P*-values) the following: *pvl*, *ent*A, the *mer* operon, *set*C
284 (staphylococcal exotoxin-like protein C), *sas*G (surface protein G), *erm*A, *aad*D, *qac*A, *xyl*R
285 (xylose repressor homolog), *aac*A-*aph*D, and *tet*M (tetracycline resistance determinant).
286 Similar to the regression analysis of cytotoxicity, these genes were associated with the CA-

287 MRSA or HA-MRSA status (Table 1), with the exception of *aadD* and *tetM*, which were
288 specific for the ST8-EMRSA2-IV and ST239-III lineages, respectively (Table S2). In multiple
289 regression analysis controlling for the CA-MRSA or HA-MRSA status of the strains, none of
290 the genes cited above were independently associated with the relative VIBL, but three other
291 genes were, namely *luk*D/E (leukocidin D/E, $P = 0.011$, F-test), *egc* (enterotoxin gene cluster,
292 $P = 0.011$) and *spl*A (serine protease-like exoprotein A, $P = 0.043$). To assess the relevance
293 of these associations, we compared the relative VIBL of the CA-MRSA and HA-MRSA strains
294 according to the presence of the *luk*D/E, *egc*, and *spl*A genes (Table 2). The differences in
295 the VIBLs were statistically significant among the CA-MRSA strains ($P < 0.01$ for all
296 differences) but not among the HA-MRSA strains. Clustering the CA-MRSA strains according
297 to the presence or absence of these genes yielded two groups, one comprised of ST8-
298 USA300-IV and ST80-IV and the other comprised of ST30-USA1100-IV. As mentioned
299 above, ST30-USA1100-IV strains had lower VIBLs than other CA-MRSA strains (Figure 1B
300 and Table S1). Hence, we cannot exclude the possibility that the association of the *luk*D/E,
301 *egc*, and *spl*A genes with strain VIBL was caused by a proxy effect in which these genes are
302 characteristic of the low-invasive ST30-USA1100-IV CA-MRSA lineage.

303

304 **Discussion**

305 The emergence of CA-MRSA as a cause of osteomyelitis has been associated with
306 an increase in both the incidence and severity of this disease. A better understanding of the
307 virulence mechanisms of CA-MRSA in osteomyelitis may help improve management
308 strategies and establish targeted therapies. Our results identify a novel virulence trait of CA-
309 MRSA by showing that the invasion of osteoblasts by such strains results in extensive cell
310 damage through a *psmα*-dependent intracellular mechanism. This virulence trait might
311 contribute to the increased severity of osteomyelitis caused by CA-MRSA relative to that
312 caused by canonical MRSA strains.

313 The invasion of osteoblasts by *S. aureus* has been extensively studied over the past
314 decade, and interpretations of the clinical significance of this phenomenon have exclusively
315 focused on chronic and indolent forms of osteomyelitis [19, 20]. More specifically, the
316 intracellular passage of bacteria has been considered a means by which *S. aureus* protects
317 itself, escapes antibiotics and the immune response of the host, and establishes a latent
318 bacterial reservoir that is potentially responsible for chronicity and recurrence [23, 24]. In
319 accordance with this interpretation, our investigations of the intracellular survival of HA-
320 MRSA strains demonstrated the ability of these strains to persist within osteoblasts without
321 causing extensive damage. Conversely, this interpretation of osteoblast invasion as an
322 underlying mechanism for chronicity and indolence does not appear relevant to severe and
323 acute CA-MRSA osteomyelitis. Indeed, our data indicate that an intracellular bacterial load of
324 one CA-MRSA cell per osteoblast is sufficient to induce the death of half of the osteoblast
325 population within 24 h (Figure 2). Hence, given the poor ability of the CA-MRSA strains to
326 persist intracellularly and the extensive damage caused to infected host cells, it is more likely
327 that osteoblast invasion by CA-MRSA is part of a pathogenesis strategy based on

328 aggression and damage rather than self-protection, a view that is consistent with previous
329 clinical observations [5, 6, 9, 10, 13].

330 Cases of CA-MRSA infections, including osteomyelitis, have been reported in several
331 different regions of the world [45, 46]. The CA-MRSA clones involved in these epidemics
332 belong to several different lineages that appear to be phylogenetically unrelated [4, 25].
333 Nevertheless, our current understanding of CA-MRSA pathogenesis is based on experiments
334 that have mostly involved strains of the USA300 lineage responsible for the US epidemic
335 [17]. As a consequence, the basis of virulence in the other CA-MRSA lineages is poorly
336 understood. The inclusion of genetically diverse strains in our experiments allowed us to
337 demonstrate that the enhanced ability to cause damage to osteoblasts was a common
338 feature among major CA-MRSA lineages, rather than a specific trait of a single lineage such
339 as USA300.

340 Alpha-toxin has previously been shown to induce apoptosis in endothelial cells
341 infected with *S. aureus* [47] and to contribute to CA-MRSA pathogenesis in a murine model
342 of pneumonia [28]. Alpha-toxin production tended to be higher, although not significantly so,
343 in CA-MRSA than in HA-MRSA strains in our experiments, but several lines of evidence
344 indicate that this toxin was not responsible for the death of the infected osteoblasts. First, we
345 failed to demonstrate any association of alpha-toxin activity with cytotoxicity; second,
346 cytotoxicity was conserved in both the non-hemolytic USA300 strain and its hemolytic
347 counterparts; and third, the 8325-4 reference strain, which had the highest alpha-toxin
348 production among our strain collection, was less cytotoxic than any of the 15 CA-MRSA
349 strains. Finally, the deletion of the *hla* gene in both the SF8300 and LAC USA300 strains
350 resulted in increased cytotoxicity toward osteoblasts (Figure 3C). The meaning of this result
351 is unclear, and further investigations are warranted to determine if alpha-toxin could protect
352 infected cells from death and to identify the mechanisms involved.

353 The contribution of PSMs to CA-MRSA virulence was first described in a murine
354 model of skin and soft tissue infection [40]. The PSM family is comprised of several proteins:
355 the delta-toxin, alpha-type PSMs 1-4, beta-type PSMs 1-2, and the SCC*mec*-encoded PSM-
356 *mec* (reviewed in [48]). Among these proteins, alpha-type PSMs were shown to be able to
357 recruit, activate, and lyse neutrophils [40], thus exhibiting a role in pathogenesis that appears
358 very similar to that of PVL. Whereas neutrophil chemotaxis and activation by PSMs occur at
359 nanomolar concentrations and involve PSM detection by the neutrophil formyl peptide
360 receptor 2 (FPR2) in vitro [49], neutrophil lysis requires micromolar concentrations of alpha-
361 type PSMs, is receptor-independent [40, 48, 49], and is thought to involve lipid membrane
362 disruption caused by the amphipathic alpha-helix structure of PSMs [40]. Interestingly, it has
363 recently been shown that human serum inhibits both the FPR2-activating and neutrophil lysis
364 properties of PSMs, casting doubt on the relevance of PSMs as extracellular toxins [50]. In
365 this context, our findings demonstrate that PSMs are major intracellular virulence factors,
366 consistent with previous observations. Indeed, *S. aureus* cells that invade non-professional
367 phagocytes such as osteoblasts remain trapped in phagosomes [51]. Hence, a sustained
368 expression of PSMs in this confined environment likely results in PSM concentrations
369 sufficient to induce phagosome membrane disruption followed by an intracytoplasmic release
370 of PSMs, ultimately leading to the death of the host cell.

371 Using Δ*agr*, Δ*sar*A, and Δ*sae*RS mutants of the CA-MRSA strain SF8300, we
372 demonstrated that only the first two regulators are involved in the intracellular cytotoxic
373 phenotype of CA-MRSA. These findings correlate with the major role of PSMs in this
374 phenotype: (i) PSM secretion by *S. aureus* is under direct control of *agr* [40]; (ii) *sar*A
375 reduces the post-secretion degradation of PSMs by downregulating the expression of the
376 aureolysin (*aur*) protease and, to a lesser extent, regulates PSM secretion by upregulating
377 *agr* [31]; and (iii) *sae*RS expression has no significant impact on PSM expression [31]. It is
378 noteworthy that *sae*RS inactivation results in an alpha-toxin-deficient phenotype [32]. Hence,

379 the lack of influence of *sae*RS on the intracellular cytotoxic phenotype in this model was also
380 consistent with our finding that alpha-toxin does not positively contribute to this form of
381 cytotoxicity.

382 DNA microarray analyses were performed to explore the potential associations
383 between a large panel of genes and the CA-MRSA or HA-MRSA status of the strains. The
384 differing distributions of genes in the two populations reflected differences in the specific
385 lineages of these strains, rather than a globally distinctive pattern between CA-MRSA and
386 HA-MRSA. Moreover, none of the screened genes was independently associated with
387 cytotoxicity in multivariate regression analysis. The lack of a significant association could be
388 related to the sample size or sampling strategy issues and does not formally eliminate the
389 possibility that an as yet-unknown CA-MRSA-specific virulence determinant contributes to
390 the intracellular cytotoxic phenotype in conjunction with PSMs. However, the fact that DNA
391 microarray analysis failed to detect genes associated with cytotoxicity supports a model in
392 which PSMs are the major factor involved in the death of infected host cells because these
393 toxins are core genome-encoded. Indeed, strain-specific differences in the expression of
394 PSMs, which rely on differences in the activity of regulatory systems such as *agr* or *sar*A, are
395 outside the scope of the DNA microarray technique used here.

396 Previous research on the basis of CA-MRSA virulence in the specific context of
397 osteomyelitis has understandably focused on the role of PVL. Cremieux et al. used a rabbit
398 model of osteomyelitis to demonstrate that PVL contributes to the severity of infection in
399 terms of bone deformation, the involvement of the surrounding soft tissue, and the systemic
400 inflammatory response [18]. These outcomes are most likely related to the potent pro-
401 inflammatory properties of PVL, including the capacity of PVL to recruit, activate, and lyse
402 immune cells at the site of infection. However, recent CA-MRSA research has emphasized
403 the remarkably complex virulence mechanisms of these pathogens, as well as the risk of

404 oversimplifying CA-MRSA virulence by considering only the individual action of a single
405 bacterial factor [17]. The multiplicity and frequent functional redundancy of CA-MRSA
406 virulence determinants are major obstacles to our understanding of CA-MRSA virulence [8],
407 and a decade of intensive research has been necessary to outline an integrated view of the
408 relative contributions of PVL and alpha-toxin to CA-MRSA pathogenesis [17]. In this context,
409 our observation that CA-MRSA strains of several genetically distinct lineages share an
410 enhanced ability to kill osteoblasts after intracellular passage through a PSM-dependent
411 mechanism adds to our knowledge of the potential pathogenesis strategies of CA-MRSA. Put
412 together, PSM-related killing of CA-MRSA-infected osteoblasts and PVL-related recruitment
413 and lysis of immune cells sketch the outlines of a new model for CA-MRSA pathogenesis in
414 osteomyelitis, in which concomitant intracellular and extracellular activity of this pathogen
415 both contribute to local tissue damage. The relative contributions of PVL-related tissue
416 damage and of PSM-related post-invasion osteoblast killing in the clinical course of CA-
417 MRSA osteomyelitis remain to be determined. To address this question, future studies
418 should focus on animal models of osteomyelitis involving Δ*pvl*, Δ*psm* and Δ*pvl-psm* CA-
419 MRSA strains, and clinical investigations should examine potential correlations between the
420 severity and acuteness of *S. aureus*-induced osteomyelitis and the strain-specific expression
421 level of PSM.

422 **Materials and Methods**

423 **Bacterial strains and growth conditions**

424 *S. aureus* strain 8325-4 was used as a reference in all experiments [52]. The strain
425 collection of the French National Reference Center for Staphylococci (FNRCS) was searched
426 for CA-MRSA and HA-MRSA isolates representative of prevalent CA-MRSA and HA-MRSA
427 genotypes in Europe according to recent epidemiological data [4, 25, 27]. Five strains of
428 each genotype were selected. The inclusion criteria were based on the molecular
429 characteristics of each strain, as available in the FNRCS database, including the sequence
430 type, SCC*mec* type, and the presence of the *pvl* and *ent*A genes. Genotype-specific
431 inclusion criteria were as follows: ST8, SCC*mec* IV, *pvl*$^+$ for the ST8-USA300-IV clone; ST80,
432 SCC*mec* IV, *pvl*$^+$ for the ST80-IV European clone; ST30, SCC*mec* IV, *pvl*$^+$ for the ST30-
433 USA1100-IV Southwest Pacific clone; ST239, SCC*mec* III for the ST239-III Brazilian clone;
434 ST228, SCC*mec* I for the ST228-I Southern German clone; ST8, SCC*mec* IV, *ent*A$^+$ for the
435 ST8-EMRSA2-IV Lyon clone [26]; and ST22, SCC*mec* IV for the ST22-EMRSA15-IV Barnim
436 clone. The lineage of each strain was subsequently confirmed using DNA microarray-based
437 assignment (data not shown) [44]. Clinical data were not considered in the strain selection
438 process because the focus of the present study was the strain genotype; in addition, the
439 number of MRSA strains in the FNRCS collection that had been isolated from documented
440 osteomyelitis cases was too low to restrict the inclusion to such strains.

441 The strains were stored at -20°C in cryotubes. For each experiment, the strains were
442 first cultivated on Columbia agar supplemented with sheep blood at 37°C for 24 h after
443 thawing. One colony was then used to inoculate brain-heart infusion (BHI) broth. In cell
444 culture infection experiments, the BHI broth was incubated overnight at 37°C, then diluted 5-
445 fold in fresh BHI and further incubated with gyratory shaking for 3 h until mid-exponential

446 phase was reached. Exponential phase cultures were preferred to stationary phase cultures
447 because bacterial adhesins involved in host cell invasion are upregulated in the former [53].

448 **Construction of allelic replacement CA-MRSA mutants**

449 The *pvl* genes *(lukS*-PV and *lukF*-PV) in the ST80-IV CA-MRSA strain HT20020209
450 was inactivated by allelic replacement. The Δ*pvl::tetM* mutant LUG1800 was obtained by
451 using pMAD, a thermo-sensitive plasmid containing a constitutively expressed ß-
452 galactosidase gene, which allows the positive selection of double crossing over by detecting
453 ß-galactosidase activity on X*gal* agar plates [54]. A 2.9-kb DNA fragment corresponding to
454 the tetracycline resistance gene *tetM* [55] was cloned into pMAD between two DNA
455 fragments generated by PCR (486 bp and 541 bp) that correspond respectively to the
456 chromosomal DNA regions upstream of *lukS*-PV (up to the start codon) and downstream of
457 *lukF*-PV (from codon 200 to the end). The resulting plasmid, pLUG934, conferred resistance
458 to ampicillin and erythromycin and contained the *lac*Z gene. pLUG934 was electroporated
459 into the *S. aureus* strain RN4220. As the plasmid from RN4220 could not be electroporated
460 into HT20020209, transformation was achieved with phage Φ11 by lysogenizing
461 RN4220/pLUG934 and transfecting HT20020209. The transformants were grown at a non-
462 permissive temperature (37°C) in the presence of 1.5 µg/mL erythromycin to select cells in
463 which the plasmid had been integrated into the chromosome by homologous recombination.
464 To favor the second recombination event, a single colony was grown at 30°C for 10
465 generations and plated at 37°C overnight. Cells that had lost the plasmid vector through a
466 double cross-over event were detected on X*gal* agar plates. PCR amplification was used to
467 confirm the loss of the *pvl* genes, which were replaced by the *tet*M gene in strain LUG1800.

468 The *pvl* genes in the ST30-USA1100-IV CA-MRSA strain BD0428 and the *hla*,
469 *psmα*1-4, *agr*A, *sar*A, and *sae*RS genes in the ST8-USA300 CA-MRSA strain SF8300 were

470 inactivated as described previously for the LAC Δ*pvl::spc* strain [36] by allelic replacement of
471 the gene(s) of interest with a spectinomycin resistance cassette.

472 **Cell culture**

473 All cell culture reagents were purchased from GIBCO (Paisley, UK). The human
474 osteoblastic cell line MG-63 was purchased from LGC Standards (Teddington, UK) and
475 grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 25 mM
476 HEPES, 10% fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin (culture
477 medium) at 37°C and 5% CO_2. The cells were subcultured twice a week and used up to
478 passage 10 after thawing.

479 **MG-63 cell invasion assay**

480 The intracellular infection of MG-63 cells was performed as described elsewhere with
481 modifications [56]. MG-63 cells were seeded at 50,000 cells/well in 24-well plates and
482 incubated at 37°C with 5% CO_2 for 48 h in culture medium. Suspensions of mid-exponential
483 phase bacterial cultures were washed, sonicated to minimize clumping, and resuspended in
484 antibiotic-free culture medium at a concentration corresponding to an MOI of 100. The
485 bacterial concentration was normalized using clone-specific regression formulas correlating
486 bacterial density (CFU/mL) with OD at 600 nm, which were established in preliminary
487 experiments. The MOIs were subsequently confirmed by plating the suspensions on agar
488 and counting the bacterial colonies. The MG-63 cells were washed twice in DMEM to remove
489 antibiotics, and normalized bacterial suspensions were added to the wells. The infected
490 cultures were incubated for 30 min at 4°C to allow the bacteria to sediment while blocking
491 internalization, and all of the cultures were simultaneously transferred to 37°C to synchronize
492 the beginning of the internalization step. After a 2 h incubation, the infected cells were
493 washed and further incubated for 1 h in culture medium containing 200 mg/L gentamicin and

494 10 mg/L lysostaphin to rapidly kill extracellular but not intracellular bacteria. Several strains
495 exhibited decreased susceptibility to gentamicin or lysostaphin when used individually (data
496 not shown), and thus the use of a gentamicin/lysostaphin combination ensured a constant
497 bactericidal activity. In experiments with time points of 24 and 48 h, the cultures were further
498 incubated for the indicated time in medium containing 40 mg/L gentamicin and 10 mg/L
499 lysostaphin. These lower concentrations resulted in the killing of bacteria cells released upon
500 host cell lysis, thus preventing these bacteria from infecting new host cells. Infected cells that
501 enter apoptosis or necrosis undergo membrane leakage, resulting in the release of the
502 cytosolic enzyme LDH into the culture supernatant, where it can be quantified. At each
503 indicated time point, the cell culture supernatant was removed, and the LDH activity was
504 assessed using a colorimetric method with a Dimension Vista automated clinical chemistry
505 analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY). Cell monolayers were washed to
506 remove antibiotics, lysed by osmotic shock in pure sterile water, and extensively pipetted to
507 achieve the full release of the internalized bacteria. Cell lysates were then sonicated to
508 minimize clumping of the bacteria and spiral-plated in duplicate on agar using a WASP
509 automated plater (AES Chemunex, Bruz, France). After 24 h of incubation, the colonies were
510 enumerated using an EasyCount automated plate reader (AES Chemunex). Due to the large
511 number of experiments required to compare the different MRSA lineages and isogenic
512 MRSA strains, the LDH release and intracellular bacterial counts were expressed relative to
513 the results of the 8325-4 reference strain in experiments involving clinical strains or relative
514 to the respective wild-type strain of each isogenic mutant in experiments involving gene
515 inactivation to control for inter-experiment variations. Conversely, experiments investigating
516 intracellular bacterial survival kinetics, as well as those investigating osteoblast mortality
517 using two representative isolates of ST80-IV (HT20020209) and ST8-EMRSA2-IV
518 (HT20040117), were conducted using three consecutive passages of MG-63 cells. The inter-

519 experiment variation was negligible in these experiments, and thus data normalization was
520 not required.

521 **Alpha-toxin quantification**

522 Alpha-toxin production by the CA-MRSA and HA-MRSA strains was assessed by
523 means of a sandwich ELISA. In brief, the wells of microtiter plates were coated with an anti-
524 alpha-toxin murine monoclonal antibody (kindly provided by GSK Biologicals) in PBS
525 overnight at room temperature. The unbound monoclonal antibody was washed out twice
526 with a blocking solution of PBS-Tween (0.05%) and milk (5 g/L), followed by incubation with
527 the blocking solution for 1 h at room temperature. Standard dilutions of recombinant alpha-
528 toxin and 24 h bacterial culture supernatant were added to duplicate wells, incubated for 1 h
529 at 37°C, and washed three times, followed by the addition of a rabbit polyclonal anti-alpha-
530 toxin antibody (GSK Biologicals). The microplates were incubated for 1 h at 37°C and
531 washed. Subsequently, a horseradish peroxidase-conjugated swine anti-rabbit polyclonal
532 antibody (DAKO SAS, Trappes, France) was added. The microplates were incubated for 1 h
533 at 37°C and washed before the addition of the tetramethylbenzidine substrate (Sigma
534 Aldrich). The reaction was stopped with H_2SO_4 after 30 min, and the plates were read at 450
535 nm in a microplate reader (Model 680, Bio-Rad). The sandwich ELISA has a lower limit of
536 detection of 2 ng/mL.

537 **DNA microarray-based genotyping**

538 Chromosomal DNA was obtained from bacterial cultures grown in BHI broth at 37°C
539 for 3 hours. After centrifugation at 3,450× g for 10 min, the bacterial pellet was resuspended
540 in a Tris-HCl buffer (1 mM) containing Triton X-100, lysostaphin (1 mg/mL), lysozyme (10
541 mg/mL), and ribonuclease A. The mixture was incubated at 37°C for 30 min, followed by the
542 addition of proteinase K and buffer AL (DNeasy kit, QIAgen). The DNA was purified using a

543 QIAcube instrument (QIAgen) according to the manufacturer's tissue lysis protocol.
544 Commercially available StaphyType DNA microarrays (Alere Technologies GmbH, Jena,
545 Germany) were used to determine the presence of 126 genes and alleles as described
546 elsewhere [57]. Microarray probes were directed toward the identification of *agr* types,
547 exotoxins, adhesins, and antibiotic resistance determinants that included genes and alleles
548 that allow the determination of the SCC*mec* types of the MRSA strains. Hybridization data
549 were used to assign strains to lineages by means of ArrayMate software (Alere
550 Technologies) by comparing the DNA microarray data of test strains to reference microarray
551 profiles stored in the ArrayMate database [44].

552 **Statistical analysis**

553 The normality of the data was assessed by visually inspecting the distributions. Two-
554 group differences were analyzed using a two-tailed Welch's *t*-test for normal data with
555 sufficient sample size ($n \geq 9$) or a two-tailed Mann-Whitney *U*-test for either non-normal data
556 or data with small sample size ($n < 9$). Multiple pairwise comparisons of the means were
557 performed using ANOVA with Tukey's HSD post-hoc test. Categorical variable frequencies
558 were analyzed using a two-tailed Fisher's exact test. Associations of numeric response
559 variables with either numeric or categorical input variables were performed by linear
560 regression. The significance of the regression coefficients was analyzed using Fisher's *F*-
561 test. Simple linear regression was performed first. Multiple linear regression was then used to
562 control for the CA-MRSA or HA-MRSA status of the strains using a one-step forward
563 selection procedure in which the CA-MRSA or HA-MRSA status was included as the first
564 input variable, followed by the inclusion of the test variable. The test variables were
565 considered to be independently associated with the response variable when their regression
566 coefficient was significant in multiple linear regression analysis. The significance threshold

567 was set at 0.05 for all tests. The statistical analyses were performed by means of R software
568 version 2.14 (The R Foundation for Statistical Computing, Vienna, Austria).

569 **Supporting Information**

570 **Table S1. A comparison of distinct lineages of CA-MRSA and HA-MRSA with respect**
571 **to cytotoxicity toward human osteoblasts, intracellular survival and alpha-toxin**
572 **production.**

573 **Table S2. Distribution of 126 genes and alleles in CA-MRSA and HA-MRSA strains as**
574 **determined using DNA microarray analysis.**

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582 **Author Contributions**

583 Conceived and designed the experiments: JPR FV FL. Performed the experiments: JPR ST
584 AS YL JR CB YB MB. Analyzed the data: JPR. Contributed reagents/materials/analysis tools:
585 TF BAD ST GL JE FV. Wrote the paper: JPR FV JE FL.

586

References

1. Lew DP, Waldvogel FA (2004) Osteomyelitis. Lancet 364(9431): 369-379.
2. Daver NG, Shelburne SA, Atmar RL, Giordano TP, Stager CE, et al. (2007) Oral step-down therapy is comparable to intravenous therapy for *Staphylococcus aureus* osteomyelitis. J Infect 54(6): 539-544.
3. Jevons MP, Coe AW, Parker MT (1963) Methicillin resistance in staphylococci. Lancet 1(7287): 904-907.
4. Deleo FR, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated meticillin-resistant *Staphylococcus aureus.* Lancet 375(9725): 1557-1568.
5. Arnold SR, Elias D, Buckingham SC, Thomas ED, Novais E, et al. (2006) Changing patterns of acute hematogenous osteomyelitis and septic arthritis: emergence of community-associated methicillin-resistant *Staphylococcus aureus.* J. Pediatr. Orthop. 26(6): 703-708.
6. Vander Have KL, Karmazyn B, Verma M, Caird MS, Hensinger RN, et al. (2009) Community-associated methicillin-resistant *Staphylococcus aureus* in acute musculoskeletal infection in children: a game changer. J Pediatr Orthop 29(8): 927-931.
7. Kaplan SL, Hulten KG, Gonzalez BE, Hammerman WA, Lamberth L, et al. (2005) Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. Clin Infect Dis 40(12): 1785-1791.
8. David MZ, Daum RS (2010) Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 23(3): 616-687.
9. Gonzalez BE, Martinez-Aguilar G, Hulten KG, Hammerman WA, Coss-Bu J, et al. (2005) Severe staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus.* Pediatrics 115(3): 642-648.

10. Martinez-Aguilar G, Avalos-Mishaan A, Hulten K, Hammerman W, Mason EO, Jr., et al. (2004) Community-acquired, methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* musculoskeletal infections in children. Pediatr Infect Dis J 23(8): 701-706.

11. Gwynne-Jones DP, Stott NS (1999) Community-acquired methicillin-resistant *Staphylococcus aureus*: a cause of musculoskeletal sepsis in children. J Pediatr Orthop 19(3): 413-416.

12. Gerber JS, Coffin SE, Smathers SA, Zaoutis TE (2009) Trends in the incidence of methicillin-resistant *Staphylococcus aureus* infection in children's hospitals in the United States. Clin Infect Dis 49(1): 65-71.

13. Bocchini CE, Hulten KG, Mason EO, Jr., Gonzalez BE, Hammerman WA, et al. (2006) Panton-Valentine leukocidin genes are associated with enhanced inflammatory response and local disease in acute hematogenous *Staphylococcus aureus* osteomyelitis in children. Pediatrics 117(2): 433-440.

14. Wilke GA, Bubeck Wardenburg J (2010) Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. Proc Natl Acad Sci U S A 107(30): 13473-13478.

15. Gouaux E (1998) Alpha-hemolysin from *Staphylococcus aureus*: an archetype of beta-barrel, channel-forming toxins. J Struct Biol 121(2): 110-122.

16. Nygaard TK, Pallister KB, Dumont AL, Dewald M, Watkins RL, et al. (2012) Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. PLoS One 7(5): e36532.

17. Otto M (2010) Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus.* Annu Rev Microbiol 64: 143-162.

18. Cremieux AC, Dumitrescu O, Lina G, Vallee C, Cote JF, et al. (2009) Panton-valentine leukocidin enhances the severity of community-associated methicillin-resistant *Staphylococcus aureus* rabbit osteomyelitis. PLoS One 4(9): e7204.

19. Ellington JK, Harris M, Hudson MC, Vishin S, Webb LX, et al. (2006) Intracellular *Staphylococcus aureus* and antibiotic resistance: implications for treatment of staphylococcal osteomyelitis. J Orthop Res 24(1): 87-93.

20. Ellington JK, Harris M, Webb L, Smith B, Smith T, et al. (2003) Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. J Bone Joint Surg Br 85(6): 918-921.

21. Jevon M, Guo C, Ma B, Mordan N, Nair SP, et al. (1999) Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. Infect Immun 67(5): 2677-2681.

22. Webb LX, Wagner W, Carroll D, Tyler H, Coldren F, et al. (2007) Osteomyelitis and intraosteoblastic *Staphylococcus aureus.* J Surg Orthop Adv 16(2): 73-78.

23. Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, et al. (2010) *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis 202(7): 1031-1040.

24. Tuchscherr L, Medina E, Hussain M, Volker W, Heitmann V, et al. (2011) *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 3(3): 129-141.

25. Tristan A, Bes M, Meugnier H, Lina G, Bozdogan B, et al. (2007) Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant *Staphylococcus aureus*, 2006. Emerg Infect Dis 13(4): 594-600.

26. Dauwalder O, Lina G, Durand G, Bes M, Meugnier H, et al. (2008) Epidemiology of invasive methicillin-resistant *Staphylococcus aureus* clones collected in France in 2006 and 2007. J Clin Microbiol 46(10): 3454-3458.

27. Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, et al. (2010) Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med 7(1): e1000215.

28. Bubeck Wardenburg J, Bae T, Otto M, Deleo FR, Schneewind O (2007) Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat Med 13(12): 1405-1406.

29. Li M, Cheung GY, Hu J, Wang D, Joo HS, et al. (2010) Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J Infect Dis 202(12): 1866-1876.

30. Morrison JM, Anderson KL, Beenken KE, Smeltzer MS, Dunman PM (2012) The Staphylococcal Accessory Regulator, SarA, is an RNA-Binding Protein that Modulates the mRNA Turnover Properties of Late-Exponential and Stationary Phase *Staphylococcus aureus* Cells. Front Cell Infect Microbiol 2: 26.

31. Zielinska AK, Beenken KE, Joo HS, Mrak LN, Griffin LM, et al. (2011) Defining the strain-dependent impact of the Staphylococcal accessory regulator (*sar*A) on the alpha-toxin phenotype of *Staphylococcus aureus.* J Bacteriol 193(12): 2948-2958.

32. Montgomery CP, Boyle-Vavra S, Daum RS (2010) Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. PLoS One 5(12): e15177.

33. Genestier AL, Michallet MC, Prevost G, Bellot G, Chalabreysse L, et al. (2005) *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. J Clin Invest 115(11): 3117-3127.

34. Said-Salim B, Mathema B, Braughton K, Davis S, Sinsimer D, et al. (2005) Differential distribution and expression of Panton-Valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* strains. J Clin Microbiol 43(7): 3373-3379.

35. Badiou C, Dumitrescu O, Croze M, Gillet Y, Dohin B, et al. (2008) Panton-Valentine leukocidin is expressed at toxic levels in human skin abscesses. Clin Microbiol Infect 14(12): 1180-1183.

36. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, et al. (2006) Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis 194(12): 1761-1770.

37. Diep BA, Chan L, Tattevin P, Kajikawa O, Martin TR, et al. (2010) Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Panton-Valentine leukocidin-induced lung inflammation and injury. Proc Natl Acad Sci U S A 107(12): 5587-5592.

38. Montgomery CP, Boyle-Vavra S, Adem PV, Lee JC, Husain AN, et al. (2008) Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. J Infect Dis 198(4): 561-570.

39. Thoendel M, Kavanaugh JS, Flack CE, Horswill AR (2011) Peptide signaling in the staphylococci. Chem Rev 111(1): 117-151.

40. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13(12): 1510-1514.

41. Kobayashi SD, Malachowa N, Whitney AR, Braughton KR, Gardner DJ, et al. (2011) Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. J Infect Dis 204(6): 937-941.

42. Rautenberg M, Joo HS, Otto M, Peschel A (2011) Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. Faseb J 25(4): 1254-1263.

43. Perret M, Badiou C, Lina G, Burbaud S, Benito Y, et al. (2012) Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner. Cell Microbiol 14(7): 1019-1036.

44. Monecke S, Slickers P, Ehricht R (2008) Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol 53(2): 237-251.

45. Kechrid A, Perez-Vazquez M, Smaoui H, Hariga D, Rodriguez-Banos M, et al. (2010) Molecular analysis of community-acquired methicillin-susceptible and resistant *Staphylococcus aureus* isolates recovered from bacteraemic and osteomyelitis infections in children from Tunisia. Clin Microbiol Infect.

46. Rozenbaum R, Sampaio MG, Batista GS, Garibaldi AM, Terra GM, et al. (2009) The first report in Brazil of severe infection caused by community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA). Braz J Med Biol Res 42(8): 756-760.

47. Haslinger-Loffler B, Kahl BC, Grundmeier M, Strangfeld K, Wagner B, et al. (2005) Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. Cell Microbiol 7(8): 1087-1097.

48. Vandenesch F, Lina G, Henry T (2012) *Staphylococcus aureus* Hemolysins, bi-component Leukocidins, and Cytolytic Peptides: A Redundant Arsenal of Membrane-Damaging Virulence Factors? Front Cell Infect Microbiol 2: 12.

49. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, et al. (2010) Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus.* Cell Host Microbe 7(6): 463-473.

50. Surewaard BG, Nijland R, Spaan AN, Kruijtzer JA, de Haas CJ, et al. (2012) Inactivation of staphylococcal phenol soluble modulins by serum lipoprotein particles. PLoS Pathog 8(3): e1002606.

51. Sinha B, Fraunholz M (2010) *Staphylococcus aureus* host cell invasion and post-invasion events. Int J Med Microbiol 300(2-3): 170-175.

52. Sjostrom JE, Lindberg M, Philipson L (1973) Competence for transfection in *Staphylococcus aureus.* J Bacteriol 113(2): 576-585.

53. Saravia-Otten P, Muller HP, Arvidson S (1997) Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by *agr* and an *agr*-independent mechanism. J Bacteriol 179(17): 5259-5263.

54. Arnaud M, Chastanet A, Debarbouille M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol 70(11): 6887-6891.

55. Nesin M, Svec P, Lupski JR, Godson GN, Kreiswirth B, et al. (1990) Cloning and nucleotide sequence of a chromosomally encoded tetracycline resistance determinant, *tet*A(M), from a pathogenic, methicillin-resistant strain of *Staphylococcus aureus.* Antimicrob Agents Chemother 34(11): 2273-2276.

56. Trouillet S, Rasigade JP, Lhoste Y, Ferry T, Vandenesch F, et al. (2011) A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells. J Microbiol Methods 86(2): 145-149.

57. Monecke S, Berger-Bachi B, Coombs G, Holmes A, Kay I, et al. (2007) Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. Clin Microbiol Infect 13(3): 236-249.

Figure Legends

Figure 1. Viable intracellular bacterial loads and host cell damage differentiate CA-MRSA and HA-MRSA strains in a model of intracellular challenge of cultured osteoblasts. Osteoblastic MG-63 cells were infected with one of 35 *S. aureus* strains belonging to 3 distinct CA-MRSA lineages (closed marks) and 4 HA-MRSA lineages (open marks) at an MOI of 100:1 and incubated for 24 h. Cytotoxicity was estimated by quantifying the LDH release by damaged cells. The infected cells were lysed, and viable intracellular bacterial counts were enumerated. All results were expressed as the n-fold change relative to the *S. aureus* 8325-4 control strain and were derived from duplicate experiments. The *P*-values were calculated using Welch's *t*-test. (A) Comparison of the relative cytotoxicity of CA-MRSA and HA-MRSA strains. (B) Comparison of the viable intracellular bacterial loads in osteoblasts infected with CA-MRSA and HA-MRSA strains. (C) Plot of relative cytotoxicity and intracellular bacterial loads, indicating differences between the CA-MRSA and HA-MRSA strains. Strains HT20020209 and HT20040117, which were included in the kinetics experiments in Figure 2, are indicated by arrows.

Figure 2. Kinetics of the intracellular passage and survival of representative CA-MRSA and HA-MRSA strains and the mortality of infected osteoblasts. The ST80-IV CA-MRSA strain HT20020209 (closed marks) and the ST8-EMRSA2-IV HA-MRSA strain HT20040117 (open marks) were used to inoculate MG-63 osteoblastic cells. The indicated *P*-values were calculated using Welch's *t*-test, and the results were derived from three independent experiments in triplicate. (A) Kinetics experiments of intracellular bacterial passage and survival. At each time point, the viable intracellular bacteria and osteoblasts were quantified to calculate the no. of viable bacteria per osteoblast. The results are shown as the means ± 95% CI. (B) The percent mortality of infected osteoblasts 24 h post-infection confirms the strong cytotoxic effect of ST80-IV *S. aureus* compared to ST8-EMRSA2-IV.

Figure 3. Impact of the major exotoxins PVL, alpha-toxin, and alpha-type PSMs and of the major regulatory systems *agr*, *sar*A, and *sae*RS on the cytotoxicity of CA-MRSA toward osteoblasts. In all experiments, cytotoxicity was estimated by quantifying LDH release by CA-MRSA-infected MG-63 osteoblastic cells at 24 h post-infection. The results were derived from three independent experiments performed in triplicate and expressed as the n-fold change in LDH release of each isogenic deletion mutant (open marks) relative to the wild-type strain (closed marks). All *P*-values were calculated using Welch's *t*-test. (A) Effect of the inactivation of *pvl* genes on the cytotoxicity of genetically diverse CA-MRSA. No significant differences were observed between the cytotoxicity of the wild-type and Δ*pvl* strains, indicating that PVL is not involved in CA-MRSA-induced cytotoxicity toward osteoblasts. (B) Impact of the inactivation of the *psmα*1-4 genes on the cytotoxicity of the USA300 CA-MRSA strain SF8300. The strain SF8300Δ*psmα*1-4 was significantly less cytotoxic than the wild-type strain, thus indicating that alpha-type PSMs are involved in the cytotoxic phenotype. (C) Impact of the inactivation of the *hla* gene on the cytotoxicity of the USA300 strains LAC and SF8300. Strains LACΔ*hla* and SF8300Δ*hla* were significantly more cytotoxic than their respective wild-type strains, suggesting a previously unidentified role of alpha-toxin as a protective agent against CA-MRSA-induced osteoblast death. (D) Impact of the inactivation of the *agr*, *sar*A, and *sae*RS genes on the cytotoxicity of strain SF8300. Strains SF8300Δ*agr* and SF8300Δ*sar*A, but not SF8300Δ*sae*RS, were significantly less cytotoxic than the wild-type strain.

Figure 4. Comparison of alpha-toxin production in CA-MRSA and HA-MRSA strains. Alpha-toxin production in 24-h bacterial culture supernatants was quantified by sandwich ELISA. (A) Alpha-toxin production was not significantly different in the CA-MRSA and HA-MRSA strains, although strong variations were observed between different lineages and within the strains of a given lineage. Horizontal lines represent median values. The *P*-value was calculated using a non-parametric Mann-Whitney *U*-test. (B) Plot of MRSA cytotoxicity toward osteoblasts against alpha-toxin production. Note that one ST8-USA300-IV CA-MRSA strain had no measurable alpha-toxin production but was more cytotoxic than any of the HA-MRSA strains. Strains HT20020209 and HT20040117, which were included in the kinetics experiments of Figure 2, are indicated by arrows.

Table 1. Genes or alleles differentially distributed in CA-MRSA and HA-MRSA strains as determined by DNA microarray analysis

Gene or allele	Protein or function	No. (%) of CA-MRSA strains				No. (%) of HA-MRSA strains					P-value[A]
		ST8-USA300-IV (n=5)	ST80-IV (n=5)	ST30-USA1100-IV (n=5)	Total (n=15)	ST239-III (n=5)	ST228-I (n=5)	ST8-EMRSA2-IV (n=5)	ST22-EMRSA15-IV (n=5)	Total (n=20)	
pvl	Panton-Valentine leukocidin	5 (100.0)	5 (100.0)	5 (100.0)	15 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.00001
*agr*III	Accessory gene regulator allele III	0 (0.0)	5 (100.0)	5 (100.0)	10 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.00002
*ent*A	Enterotoxin A	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)	5 (100.0)	5 (100.0)	0 (0.0)	13 (65.0)	0.00006
mer	Mercury resistance operon	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	5 (100.0)	1 (20.0)	0 (0.0)	11 (55.0)	0.00055
*erm*A	Erythromycin resistance	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (80.0)	5 (100.0)	2 (40.0)	0 (0.0)	11 (55.0)	0.00055
*qac*A	Quaternary ammonium cations resistance	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (80.0)	5 (100.0)	1 (20.0)	0 (0.0)	10 (50.0)	0.00157
*aac*A-*aph*D	Aminoglycoside resistance	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (80.0)	5 (100.0)	0 (0.0)	0 (0.0)	9 (47.4)	0.00186
*set*C	Staphylococcal exotoxin-like protein C	5 (100.0)	5 (100.0)	0 (0.0)	10 (66.7)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	20 (100.0)	0.00925
*sas*G	Surface protein G	5 (100.0)	5 (100.0)	0 (0.0)	10 (66.7)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	20 (100.0)	0.00925
*et*D	Exfoliative toxin gene D	0 (0.0)	5 (100.0)	0 (0.0)	5 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.00925
*edin*B	Epidermal cell differentiation inhibitor B	0 (0.0)	5 (100.0)	0 (0.0)	5 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.00925
*agr*I	Accessory gene regulator allele I	5 (100.0)	0 (0.0)	0 (0.0)	5 (33.3)	5 (100.0)	0 (0.0)	5 (100.0)	5 (100.0)	15 (75.0)	0.01923
cap 8	Capsular type 8	0 (0.0)	5 (100.0)	5 (100.0)	10 (66.7)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (25.0)	0.01923
cap 5	Capsular type 5	5 (100.0)	0 (0.0)	0 (0.0)	5 (33.3)	0 (0.0)	5 (100.0)	5 (100.0)	5 (100.0)	15 (75.0)	0.01923
*far*1	Fusidic acid resistance	0 (0.0)	4 (80.0)	0 (0.0)	4 (26.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.02607
*arc*A-SCC	Arginine deiminase (ACME locus)	4 (80.0)	0 (0.0)	0 (0.0)	4 (26.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.02607
*fnb*B	Fibronectin-binding protein B	5 (100.0)	5 (100.0)	5 (100.0)	15 (100.0)	5 (100.0)	2 (40.0)	5 (100.0)	2 (40.0)	14 (70.0)	0.02696
*xyl*R	xylose repressor homolog	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (25.0)	0.04470

[A]*P*-values for differences between CA-MRSA and HA-MRSA strains were calculated using a two-tailed Fisher's exact test.

Table 2. Comparison of the viable intracellular bacterial load (VIBL) of CA-MRSA and HA-MRSA strains according to the presence of the *luk*D/E, *egc*, and *spl*A loci.

	CA-MRSA			HA-MRSA		
Locus	Genetic background and no. of positive strains	Relative VIBL of positive vs. negative strains (mean and 95% confidence interval)	P-value[A]	Genetic background and no. of positive strains	Relative VIBL of positive vs. negative strains (mean and 95% confidence interval)	P-value[A]
*luk*D/E	ST8-USA300 (5), ST80 (5)	0.88 [0.69-1.06] vs. 0.42 [0.32-0.51]	0.008	ST239 (5), ST228 (3), ST8-EMRSA2 (5)	2.70 [2.29-3.10] vs. 2.12 [1.68-2.56]	0.097
egc	ST30 (5)	0.42 [0.32-0.51] vs. 0.88 [0.69-1.06]	0.008	ST228 (5), ST22 (5)	2.22 [1.89-2.55] vs. 2.77 [2.25-3.29]	0.123
*spl*A	ST8-USA300 (5), ST80 (5)	0.88 [0.69-1.06] vs. 0.42 [0.32-0.51]	0.008	ST239 (5), ST228 (5), ST8-EMRSA2 (5)	2.61 [2.22-3.00] vs. 2.16 [1.68-2.64]	0.197

[A] *P*-values for the differences between strains positive and negative for each gene were calculated using a two-tailed Mann-Whitney *U*-test.

FIGURE 1.

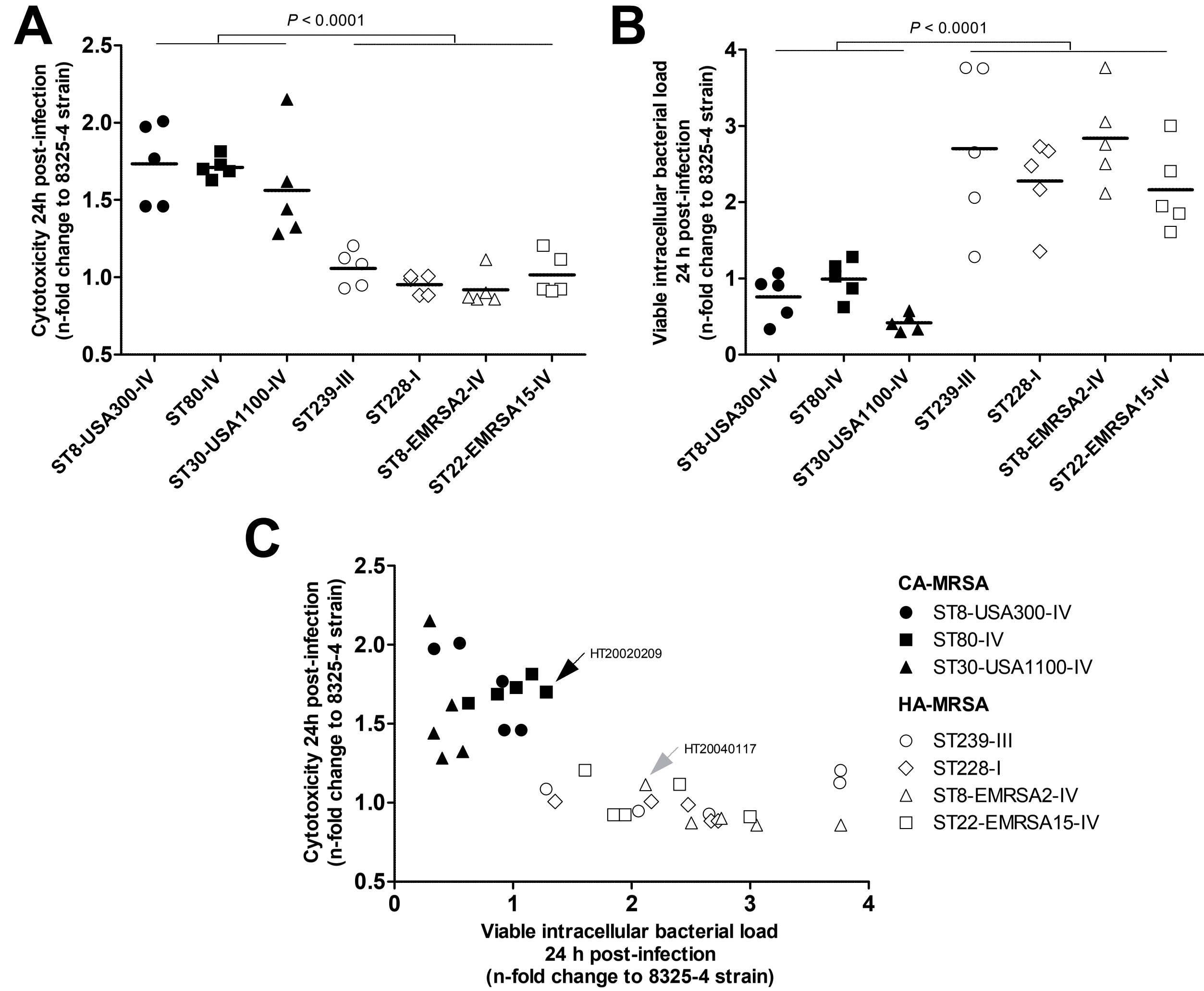


FIGURE 2.

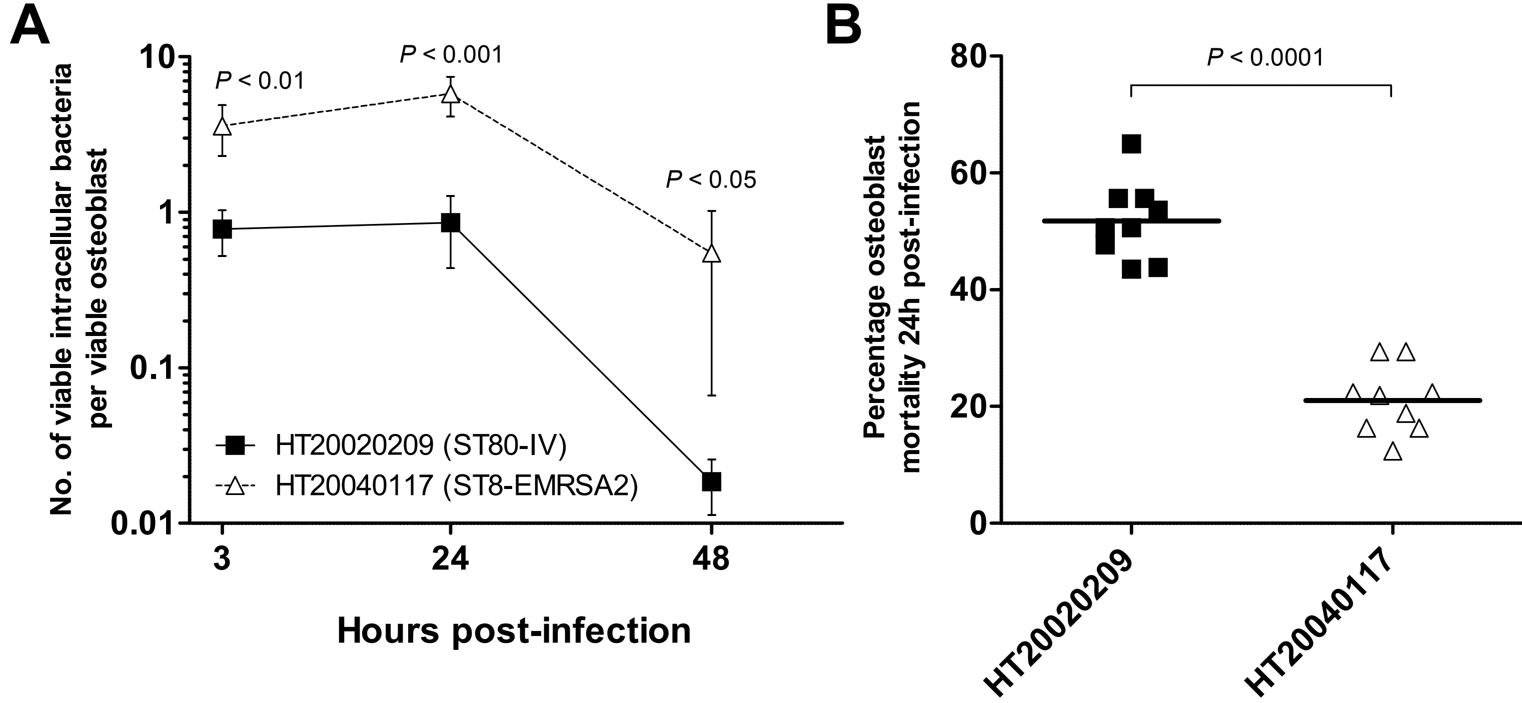
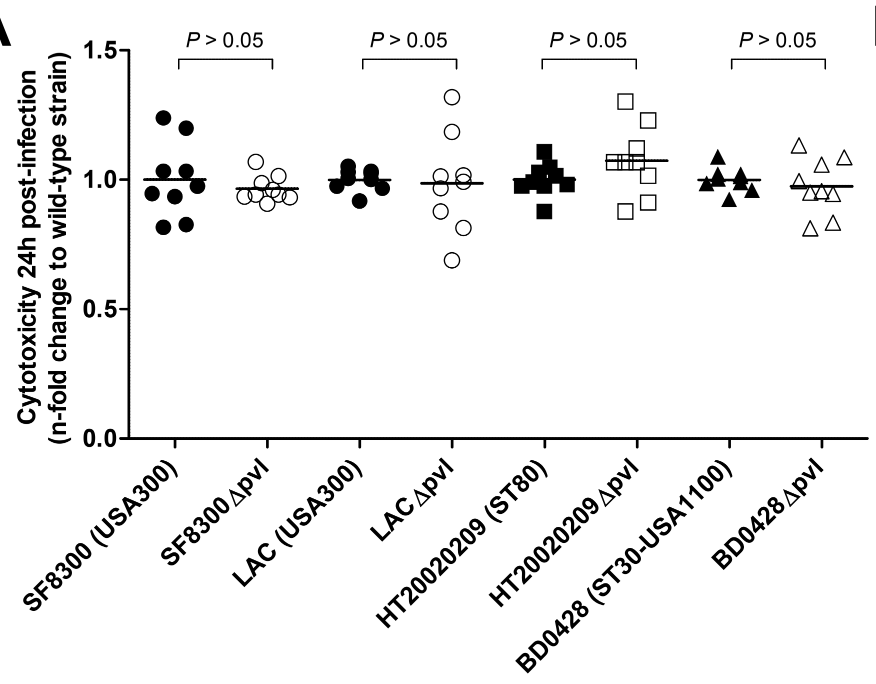
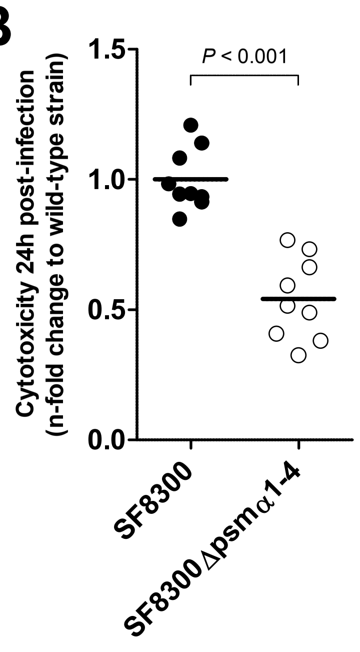
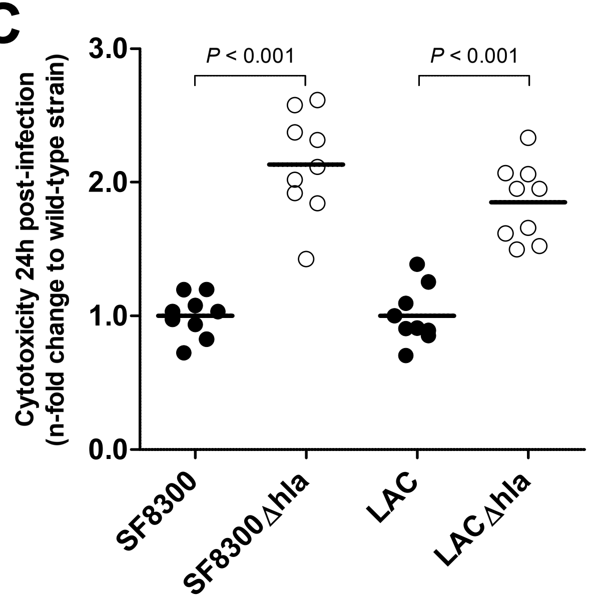


FIGURE 3.







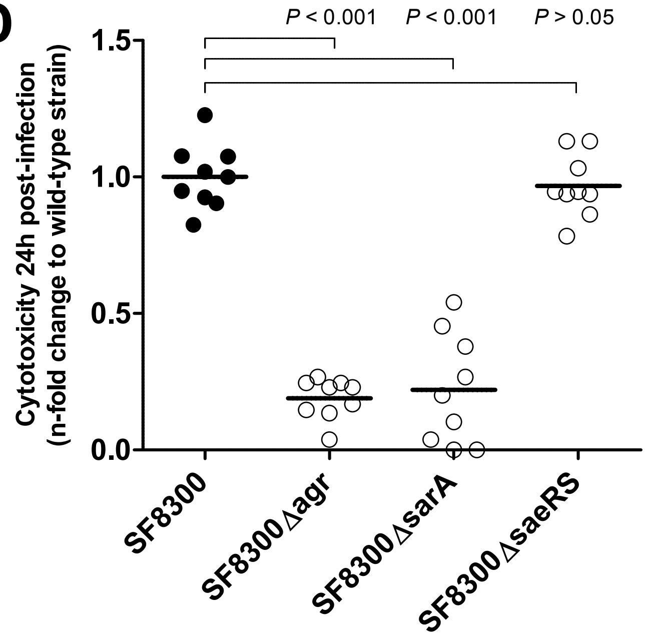
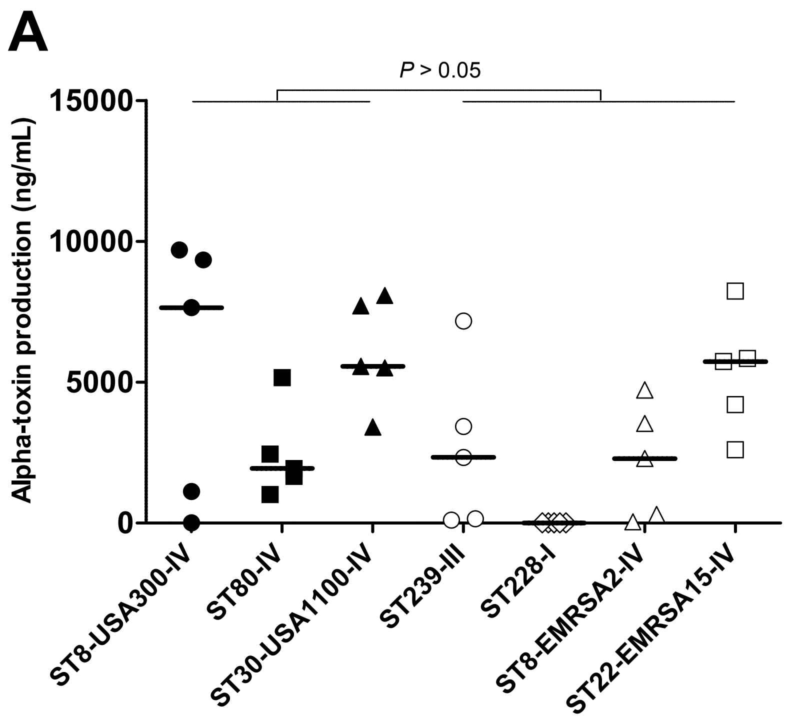


FIGURE 4.



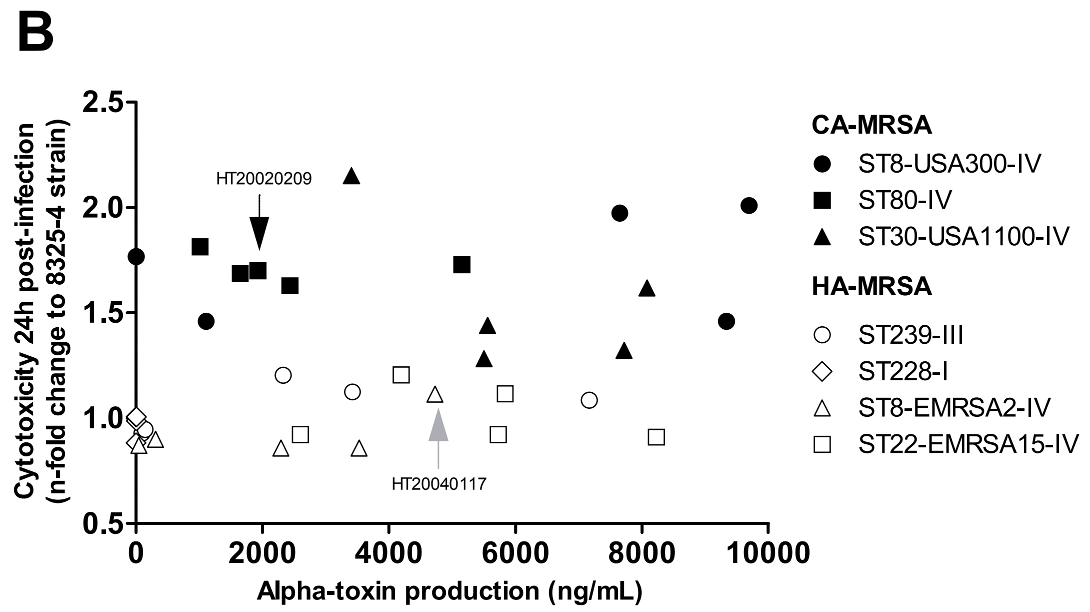


Table S1. A comparison of distinct lineages of CA-MRSA and HA-MRSA with respect to cytotoxicity toward human osteoblasts, intracellular survival, and alpha-toxin production.

Lineage	LDH release by infected osteoblasts (mean n-fold change to 8325-4 strain [95%CI])	Viable intracellular bacterial load (mean n-fold change to 8325-4 strain [95%CI])	Alpha-toxin production (median ng/ml [IQR])
ST8-USA300-IV (n=5)	1.73 [1.40-2.07]	0.76 [0.38-1.14]	7650 [1110-9337]
ST80-IV (n=5)	1.71 [1.63-1.80]	0.99 [0.67-1.31]	1929 [1649-2434]
ST30-USA1100-IV (n=5)	1.56 [1.12-2.00]	0.42 [0.28-0.56]	5556 [5503-7715]
CA-MRSA (total, n=15)	1.67 [1.53-1.81]	0.72 [0.54-0.91]	5153 [1790-7683]
ST239-III (n=5)	1.06 [0.91-1.21]	2.70 [1.36-4.05]	2327 [143-3424]
ST228-I (n=5)	0.95 [0.87-1.03]	2.28 [1.58-2.98]	4 [3-8]
ST8-EMRSA2-IV (n=5)	0.92 [0.78-1.06]	2.84 [2.07-3.61]	2293 [305-3529]
ST22-EMRSA15-IV (n=5)	1.02 [0.85-1.18]	2.16 [1.48-2.85]	5729 [4193-5839]
HA-MRSA (total, n=20)	0.99 [0.93-1.04]	2.50 [2.15-2.84]	2310 [36-4326]

Table S2. Distribution of 126 genes and alleles in CA-MRSA and HA-MRSA strains as determined using DNA microarray analysis

Gene	No. (%) of CA-MRSA strains				No. (%) of HA-MRSA strains					P-value*
	ST8-USA300-IV (n=5)	ST80-IV (n=5)	ST30-USA1100-IV (n=5)	**Total (n=15)**	ST239-III (n=5)	ST228-I (n=5)	ST8-EMRSA2-IV (n=5)	ST22-EMRSA15-IV (n=5)	**Total (n=20)**	
SPECIES MARKER										
Ribos. STAU	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
gapA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
katA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
CoA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
nuc1	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
spa	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
sbi	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
REGULATORY GENES										
sarA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
saeS	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
agrI	5 (100.0)	0 (0.0)	0 (0.0)	**5 (33.3)**	5 (100.0)	0 (0.0)	5 (100.0)	5 (100.0)	**15 (75.0)**	0.01923
agrII	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
agrIII	0 (0.0)	5 (100.0)	5 (100.0)	**10 (66.7)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.00002
agrIV	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
hld	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
METHICILLIN RESISTANCE AND SCC*mec* TYPING										
mecA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
delta_mecR	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
ugpQ	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
ccrB-1	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
plsSCC (COL)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
ccrA-2	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	0 (0.0)	0 (0.0)	5 (100.0)	5 (100.0)	**10 (50.0)**	0.00157
kdpA-SCC	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
mecI	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
mecR	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
xylR	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
ccrA-3	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
merA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	5 (100.0)	1 (20.0)	0 (0.0)	**11 (55.0)**	0.00055
ccrC (85-2082)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
RESISTANCE : PENICILLINASE										
blaZ	4 (80.0)	4 (80.0)	5 (100.0)	**13 (86.7)**	5 (100.0)	5 (100.0)	4 (80.0)	5 (100.0)	**19 (95.0)**	0.56455
RESISTANCE : MLS-ANTIBIOTICS										
ermA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	4 (80.0)	5 (100.0)	2 (40.0)	0 (0.0)	**11 (55.0)**	0.00055
ermB	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
ermC	1 (20.0)	1 (20.0)	0 (0.0)	**2 (13.3)**	0 (0.0)	0 (0.0)	2 (40.0)	3 (60.0)	**5 (25.0)**	0.67217
linA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
msrA	3 (60.0)	0 (0.0)	0 (0.0)	**3 (20.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.06952
mefA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
mpbBM	3 (60.0)	0 (0.0)	0 (0.0)	**3 (20.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.06952
vatA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vatB	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vga	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vgaA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vgb	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
RESISTANCE : AMINOGLYCOSIDES										
aacA-aphD	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	4 (80.0)	5 (100.0)	0 (0.0)	0 (0.0)	**9 (45.0)**	0.00427
aadD	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	5 (100.0)	0 (0.0)	**5 (25.0)**	0.05701
aphA3	3 (60.0)	5 (100.0)	0 (0.0)	**8 (53.3)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.15672
RESISTANCE : MISCELLANEOUS GENES										
sat	3 (60.0)	5 (100.0)	0 (0.0)	**8 (53.3)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.15672
dfrA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
far1	0 (0.0)	4 (80.0)	0 (0.0)	**4 (26.7)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.02607
mupR	1 (20.0)	0 (0.0)	0 (0.0)	**1 (6.7)**	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	**1 (5.0)**	1.00000
tetK	0 (0.0)	3 (60.0)	0 (0.0)	**3 (20.0)**	3 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)	**3 (15.0)**	1.00000
tetM	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.05701
cat	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
cfr	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
fexA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
RESISTANCE : EFFLUX SYSTEMS										
qacA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	4 (80.0)	5 (100.0)	1 (20.0)	0 (0.0)	**10 (50.0)**	0.00157
qacC	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
RESISTANCE : GLYCOPEPTIDES										
vanA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vanB	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
vanZ	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
VIRULENCE : TOXIC SHOCK TOXIN										
tst1 (consensus)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
tst1 ("human" allele)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
tst1 ("bovine" allele)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
VIRULENCE : ENTEROTOXINS										
entA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	3 (60.0)	5 (100.0)	5 (100.0)	0 (0.0)	**13 (65.0)**	0.00006
entA (320E)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
entA (N315) / *entP*	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
entB	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
entC	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	**1 (5.0)**	1.00000
entD	1 (20.0)	0 (0.0)	0 (0.0)	**1 (6.7)**	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)	**3 (15.0)**	0.61898
entE	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
entG	0 (0.0)	0 (0.0)	4 (80.0)	**4 (26.7)**	0 (0.0)	3 (60.0)	0 (0.0)	5 (100.0)	**8 (40.0)**	0.48854
entH	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
entI	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	0 (0.0)	3 (60.0)	0 (0.0)	5 (100.0)	**8 (40.0)**	0.73720
entJ	1 (20.0)	0 (0.0)	0 (0.0)	**1 (6.7)**	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)	**3 (15.0)**	0.61898
entK	5 (100.0)	0 (0.0)	0 (0.0)	**5 (33.3)**	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	**4 (20.0)**	0.45050
entL	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	**1 (5.0)**	1.00000
entM	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	0 (0.0)	3 (60.0)	0 (0.0)	5 (100.0)	**8 (40.0)**	0.73720
entN (consensus)	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	0 (0.0)	3 (60.0)	0 (0.0)	5 (100.0)	**8 (40.0)**	0.73720
entO	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	0 (0.0)	5 (100.0)	0 (0.0)	5 (100.0)	**10 (50.0)**	0.49160
entQ	4 (80.0)	0 (0.0)	0 (0.0)	**4 (26.7)**	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	**4 (20.0)**	0.70027
entR	1 (20.0)	0 (0.0)	0 (0.0)	**1 (6.7)**	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)	**3 (15.0)**	0.61898
entU	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	0 (0.0)	3 (60.0)	0 (0.0)	5 (100.0)	**8 (40.0)**	0.73720
entCM14 probe1	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000

No. (%) of CA-MRSA strains No. (%) of HA-MRSA strains

Gene	ST8-USA300-IV (n=5)	ST80-IV (n=5)	ST30-USA1100-IV (n=5)	**Total (n=15)**	ST239-III (n=5)	ST228-I (n=5)	ST8-EMRSA2-IV (n=5)	ST22-EMRSA15-IV (n=5)	**Total (n=20)**	P-value*
VIRULENCE : HLG AND LEUKOCIDINS										
lukF	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	2 (40.0)	**17 (85.0)**	0.24370
lukS	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	4 (80.0)	**19 (95.0)**	1.00000
lukS (ST22+ST45)	1 (20.0)	4 (80.0)	4 (80.0)	**9 (60.0)**	0 (0.0)	4 (80.0)	5 (100.0)	5 (100.0)	**14 (70.0)**	0.72102
hlgA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	4 (80.0)	**19 (95.0)**	1.00000
lukF-PV	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	<0.00001
lukS-PV	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	<0.00001
lukF-PV (P83)	0 (0.0)	1 (20.0)	0 (0.0)	**1 (6.7)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.42857
lukD	5 (100.0)	5 (100.0)	0 (0.0)	**10 (66.7)**	5 (100.0)	3 (60.0)	5 (100.0)	0 (0.0)	**13 (65.0)**	1.00000
lukE	4 (80.0)	5 (100.0)	0 (0.0)	**9 (60.0)**	5 (100.0)	3 (60.0)	5 (100.0)	0 (0.0)	**13 (65.0)**	1.00000
hla	4 (80.0)	5 (100.0)	5 (100.0)	**14 (93.3)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	0.42857
VIRULENCE : HLB-CONVERTING PHAGES										
sak	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	4 (80.0)	5 (100.0)	5 (100.0)	5 (100.0)	**19 (95.0)**	1.00000
chp	4 (80.0)	0 (0.0)	5 (100.0)	**9 (60.0)**	1 (20.0)	0 (0.0)	0 (0.0)	5 (100.0)	**6 (30.0)**	0.09660
scn	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	4 (80.0)	5 (100.0)	5 (100.0)	5 (100.0)	**19 (95.0)**	1.00000
VIRULENCE : EXFOLIATIVE TOXINS										
etA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
etB	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
etD	0 (0.0)	5 (100.0)	0 (0.0)	**5 (33.3)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.00925
VIRULENCE : EPITHELIAL DIFFERENTIATION INHIBITOR										
edinA	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
edinB	0 (0.0)	5 (100.0)	0 (0.0)	**5 (33.3)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.00925
edinC	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
VIRULENCE : ACME LOCUS										
arcA-SCC	4 (80.0)	0 (0.0)	0 (0.0)	**4 (26.7)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0.02607
VIRULENCE : PROTEASES										
aur (consensus)	3 (60.0)	4 (80.0)	4 (80.0)	**11 (73.3)**	0 (0.0)	2 (40.0)	5 (100.0)	3 (60.0)	**10 (50.0)**	0.29580
aur (Other than MRSA252)	4 (80.0)	5 (100.0)	0 (0.0)	**9 (60.0)**	0 (0.0)	5 (100.0)	5 (100.0)	0 (0.0)	**10 (50.0)**	0.73378
aur (MRSA252)	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	5 (100.0)	0 (0.0)	0 (0.0)	5 (100.0)	**10 (50.0)**	0.49160
splA	5 (100.0)	5 (100.0)	0 (0.0)	**10 (66.7)**	5 (100.0)	5 (100.0)	5 (100.0)	0 (0.0)	**15 (75.0)**	0.71180
splB	5 (100.0)	5 (100.0)	0 (0.0)	**10 (66.7)**	5 (100.0)	5 (100.0)	5 (100.0)	0 (0.0)	**15 (75.0)**	0.71180
splE	5 (100.0)	0 (0.0)	5 (100.0)	**10 (66.7)**	4 (80.0)	0 (0.0)	5 (100.0)	0 (0.0)	**9 (45.0)**	0.30636
CAPSULE- AND BIOFILM-ASSOCIATED GENES										
cap 1	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	1.00000
cap 5	5 (100.0)	0 (0.0)	0 (0.0)	**5 (33.3)**	0 (0.0)	5 (100.0)	5 (100.0)	5 (100.0)	**15 (75.0)**	0.01923
cap 8	0 (0.0)	5 (100.0)	5 (100.0)	**10 (66.7)**	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	**5 (25.0)**	0.01923
icaA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
icaC	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
icaD	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
ADHESION FACTORS / MSCRAMM GENES										
bbp	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	3 (60.0)	5 (100.0)	**18 (90.0)**	0.49580
clfA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
clfB	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
cna	0 (0.0)	0 (0.0)	5 (100.0)	**5 (33.3)**	5 (100.0)	0 (0.0)	0 (0.0)	5 (100.0)	**10 (50.0)**	0.49160
ebh (consensus)	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	0 (0.0)	**15 (75.0)**	0.05701
ebpS	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
eno	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
fnbA	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	4 (80.0)	5 (100.0)	5 (100.0)	5 (100.0)	**19 (95.0)**	1.00000
fnbB	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	2 (40.0)	5 (100.0)	2 (40.0)	**14 (70.0)**	0.02696
map	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	3 (60.0)	**18 (90.0)**	0.49580
sasG	5 (100.0)	5 (100.0)	0 (0.0)	**10 (66.7)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	0.00925
sdrC	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000
sdrD	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	3 (60.0)	5 (100.0)	**18 (90.0)**	0.49580
vwb	5 (100.0)	5 (100.0)	5 (100.0)	**15 (100.0)**	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	**20 (100.0)**	1.00000

*P-values for differences between CA-MRSA and HA-MRSA strains were calculated using a two-tailed Fisher's exact test.

IV. Troisième Publication : Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*

Jean-Philippe Rasigade, Abdelmalek Moulay, Yannick Lhoste, Anne Tristan, Michèle Bes, François Vandenesch, Jérôme Etienne, Gérard Lina, Frédéric Laurent, Oana Dumitrescu.

***BMC Microbiology* 2011 ;11 : 263**

L'invasion des cellules eucaryotes par *S. aureus* est dépendante de protéines de surface fixant la fibronectine, les FnBP. Cette invasion semble un jouer un rôle important dans certaines pathologies, notamment les IOA, soit en permettant la constitution d'un réservoir bactérien latent, à l'abri des antibiotiques et de la réponse immunitaire de l'hôte, soit en permettant à certaines souches hypervirulentes de *S. aureus* de tuer la cellule infectée. Nous avons exploré l'impact de différents antibiotiques à dose sub-inhibitrice sur l'expression des FnBP, l'adhésion à la fibronectine et l'invasion des ostéoblastes humains. Cet objectif découlait d'une part de la présence de faibles concentrations d'antibiotiques dans le tissu osseux au cours de la prophylaxie pré-opératoire ou du traitement des IOA, et d'autre part de l'observation que certains antibiotiques à dose sub-inhibitrice modulent, positivement ou négativement, l'expression des exotoxines chez *S. aureus* (Herbert *et al.*, 2001; Dumitrescu *et al.*, 2007; Stevens *et al.*, 2007). Nous avons ainsi émis l'hypothèse que l'expression des FnBP pouvait être modulée par les antibiotiques de façon analogue aux exotoxines.

Grâce à une approche par RT-PCR quantitative, nous avons montré que le niveau transcriptionnel des gènes *fnb*A et *fnb*B était influencé par les antibiotiques ; nous avons également observé que cette modulation avait un impact phénotypique sur l'adhésion de *S. aureus* à la fibronectine. Cependant, cette modification phénotypique n'a pas été confirmée en termes d'adhésion et d'invasion des ostéoblastes humains par *S. aureus* dans le modèle développé au sein du laboratoire. Il est probable que les interactions complexes entre *S. aureus* et les cellules eucaryotes font intervenir des systèmes redondants et que la modification quantitative et partielle de l'expression des FnBP n'est pas suffisante à elle seule pour induire une modification de la capacité d'invasion cellulaire.

Contribution personnelle au travail : réalisation des expériences ex vivo, analyse et interprétation des données, rédaction et révision du manuscrit.

Contributions majeures non réalisées par l'auteur : G. Lina et O. Dumitrescu ont apporté la problématique, conçu les expériences de RT-PCR quantitative et participé de façon substantielle à la rédaction du manuscrit; A. Moulay a réalisé les expériences de RT-PCR quantitative et d'adhésion à la fibronectine in vitro.

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RESEARCH ARTICLE Open Access

Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*

Jean Philippe Rasigade[1,2], Abdelmalek Moulay[1,2], Yannick Lhoste[1,2], Anne Tristan[1,2], Michele Bes[1,2], François Vandenesch[1,2], Jerome Etienne[1,2], Gerard Lina[1,2], Frederic Laurent[1,2] and Oana Dumitrescu[1,2,3*]

Abstract

Background: *Staphylococcus aureus* is a well-armed pathogen prevalent in severe infections such as endocarditis and osteomyelitis. Fibronectin-binding proteins A and B, encoded by *fnb*A/B, are major pathogenesis determinants in these infections through their involvement in *S. aureus* adhesion to and invasion of host cells. Sub-minimum inhibitory concentrations (sub-MICs) of antibiotics, frequently occurring *in vivo* because of impaired drug diffusion at the infection site, can alter *S. aureus* phenotype. We therefore investigated their impact on *S. aureus* fibronectin-mediated adhesiveness and invasiveness.

Methods: After *in vitro* challenge of *S. aureus* 8325-4 and clinical isolates with sub-MICs of major anti-staphylococcal agents, we explored *fnb*A/B transcription levels, bacterial adhesiveness to immobilised human fibronectin and human osteoblasts in culture, and bacterial invasion of human osteoblasts.

Results: Oxacillin, moxifloxacin and linezolid led to the development of a hyper-adhesive phenotype in the fibronectin adhesion assay that was consistent with an increase in *fnb*A/B transcription. Conversely, rifampin treatment decreased fibronectin binding in all strains tested without affecting *fnb*A/B transcription. Gentamicin and vancomycin had no impact on fibronectin binding or *fnb*A/B transcription levels. Only oxacillin-treated *S. aureus* displayed a significantly increased adhesion to cultured osteoblasts, but its invasiveness did not differ from that of untreated controls.

Conclusion: Our findings demonstrate that several antibiotics at sub-MICs modulate fibronectin binding in *S. aureus* in a drug-specific fashion. However, hyper- and hypo- adhesive phenotypes observed in controlled *in vitro* conditions were not fully confirmed in whole cell infection assays. The relevance of adhesion modulation during *in vivo* infections is thus still uncertain and requires further investigations.

Background

Staphylococcus aureus is a prevalent and dangerous pathogen in humans, causing a wide range of infections. The initial step of suppurative infections, such as infective endocarditis or osteomyelitis, involves bacterial adhesion to the extracellular matrix and cell surface of the host. Several microbial factors involved in this adherence are present in *S. aureus* [1]. Among these factors, collectively designated as microbial surface components recognising adhesive matrix molecules (MSCRAMMs), fibronectin-binding proteins A and B (FnBPA/B) play a major role in *S. aureus* pathogenicity through their ability to bind fibronectin and fibrinogen and to initiate integrin-mediated intracellular uptake of the bacteria by non-professional phagocytes such as endothelial cells or osteoblasts [2,3]. The invasion of host cells by *S. aureus* eventually leads to the formation of an intracytoplasmic reservoir, where bacteria remain protected from the action of cell- and antibody-mediated immune response and from that of most antimicrobial agents. This bacterial sanctuarisation makes successful treatment even more challenging and paves the way for infection relapse [4].

* Correspondence: oana.dumitrescu@chu-lyon.fr
[1]INSERM, U851, Lyon 69008, France
Full list of author information is available at the end of the article

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A peculiar difficulty to be faced in treating deep-seated infections is the risk of impaired diffusion of antimicrobial agents at the infection site, where they would only achieve sub-inhibitory concentrations. *S. aureus* strains challenged with such antibiotic concentrations have been shown to exhibit altered phenotypes depending on the molecule tested, including down- or up-regulation of virulence factor expression. For example, beta-lactams enhance the secretion of virulence factors such as the Panton-Valentine leukocidin and alpha haemolysin, while clindamycin or linezolid exert an inhibitory effect [5-8]. However, most studies on the antibiotic-mediated modulation of protein expression by *S. aureus* have focused on secreted exotoxins, and less is known about this modulation with respect to MSCRAMMS, including FnBPA/B.

In the present study, we aimed to investigate the impact of sub-inhibitory concentrations of major anti-staphylococcal agents on the adhesion and invasion phenotypes of *S. aureus*. After *in vitro* challenge of *S. aureus* reference strain 8325-4 and clinical isolates with antibiotics, we explored the following: (i) mRNA expression levels of the *fnb*A and *fnb*B genes, which encode FnBPA and B, respectively; (ii) bacterial adhesiveness to immobilised human fibronectin and human osteoblasts in culture; and (iii) bacterial invasion of human osteoblasts.

Methods

Bacterial strains

The bacterial strains used in this study are summarised in Table 1. Laboratory strain 8325-4 and its Δ*fnb*A/B derivative DU5883 were used as a control for *fnb*A/B [9]. Clinical isolates were characterised for the presence of the *fnb*A, *fnb*B, *agr*1-4 and *mec*A genes by PCR as previously described [10,11], and MLST was performed as described by Enright et al. to identify their genetic background [12].

Antibiotics and MIC determination

The antibiotics used in this study were as follows: oxacillin, gentamicin, clindamycin, rifampicin and vancomycin purchased from Sigma-Aldrich (L'Isle d'Abeau, France); linezolid provided by Pfizer (Amboise, France); and moxifloxacin provided by Bayer (Wuppertal, Germany). Minimal inhibitory concentrations were determined by broth microdilution assay as recommended by the Clinical Laboratory Standards Institute (CLSI) standards [13].

Table 1 Characterisation of bacterial strains used in this study

Strain	Type	*fnb*A	*fnb*B	*mec*A	*agr*	ST[a]
8325-4	laboratory strain	+	+	-	1	8
DU 5883	8325-4 Δ*fnb*A/B [9]	-	-	-	1	8
ST2008 1028	clinical isolate, osteomyelitis	+	+	-	3	30
ST2008 0563	clinical isolate, osteomyelitis	+	+	-	1	8
HT2001 0390	clinical isolate, endocarditis	+	+	-	3	1
HT2001 0594	clinical isolate, endocarditis	+	+	-	2	15

[a]Sequence Type (ST) was determined as described by Enright et al. [12]

Bacterial cultures

The strains were cultured on trypticase blood agar plates and incubated overnight at 37°C. Isolated colonies were resuspended in 5 ml brain heart infusion (BHI) in glass tubes (AES Chemunex France) and adjusted to 0.5 McFarland turbidity, corresponding to 10^8 CFU/ml, as confirmed by bacterial count. Bacterial suspensions were cultivated at 37°C with 300 rpm gyratory shaking. After 1 h, antibiotics were added to the culture medium at a concentration of half the MIC, and the incubation was continued for 2 additional hours to reach the mid-exponential phase. McFarland turbidity was measured at the end of the incubation step to determine the impact of antibiotics treatment on bacterial density. Aliquots were then taken, and cellular pellets were prepared as described below for total RNA extraction, the microplate adhesion assay, and the whole cell adhesion and invasion assay.

Relative quantitative RT-PCR

Aliquots of 1 mL of the *S. aureus* 8325-4 cultures were centrifuged at 13,000 g, and the pellets were washed with 1 mL of 10 mM Tris buffer and adjusted to an optical density at 600 nm (OD600) of 1, corresponding to approximately 1×10^9 *S. aureus* cells/mL. One mL of adjusted and washed bacterial suspension was centrifuged at 13,000 g, and the pellets were treated with lysostaphin (Sigma-Aldrich) at a final concentration of 200 mg/L. The total RNA of the pellets was then purified using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA yield was assessed by UV absorbance, and 1 microgram of total RNA was reverse transcribed using the Reverse Transcription System (Promega) with random primers, as recommended by the provider. The resulting cDNA was used as the template for real-time amplification of *gyr*B, *fnb*A and *fnb*B using specific primers (Table 2). The relative amounts of the *fnb*A and *fnb*B amplicons were determined by quantitative PCR relative to a *gyr*B internal standard, as described elsewhere [14]. The calibrators in our study were the transcripts from the *S. aureus* 8325-4 strain grown without antibiotics, normalised with respect to *gyr*B transcription level. *gyr*B expression was not modified by sub-inhibitory antibiotics, thus allowing its use as an internal control. The relative fold changes in the *fnb*A and *fnb*B expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using the RealQuant software (Roche Diagnostics).

Table 2 Primers used for qRT-PCR in this study

Primer name	5' 3' sequence	Reference
*gyr*B F	GGTGGCGACTTTGATCTAGC	[14]
*gyr*B R	TTATACAACGGTGGCTGTGC	[14]
*fnb*A F	ATTGAGACATTTAATAAAGCGA	[15]
*fnb*A R	TTTTGAATAATCGGACCATT	[15]
*fnb*B F	CACCGAAAACTGTGCAAGCA	[16]
*fnb*B R	TTCCTGTAGTTTCCTTATCAGCAACTT	[16]

Microplate adhesion assay

Flat-bottom 96-well plates (Immuno Nunc) were coated with 50 μg/mL plasma human fibronectin (Sigma-Aldrich) and incubated overnight at 4°C. Coated plates were inoculated with 200 μL per well of bovine serum albumin BSA and incubated for 20 min at 37°C, and then each well was washed 3 times.

Aliquots of the bacterial cultures described above were centrifuged at 13,000 g for 10 min, and the cellular pellets were washed and resuspended in PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich). Bacterial suspensions were adjusted to an OD600 of 1, corresponding to approximately 1×10^9 *S. aureus* cells/mL. One hundred μL of each bacterial suspension was incubated in 3 different wells of the fibronectin-coated plate for 45 min at 37°C with mild shaking. Each well was washed 3 times with PBS to remove non-adherent bacteria. Adherent bacteria were fixed with glutaraldehyde (2.5% v/v in 0.1 mol/L PBS) for 2 h at 4°C and then stained with crystal violet (0.1% m/v) for 30 min at room temperature. Excess stain was rinsed off with Triton X100 solution (0.2% v/v, H_2O), and the plates were dried at room temperature. Bacterial adhesion to fibronectin was assessed spectrophotometrically (Spectrophotometer MR5000, Dynatec) by determining the optical density at 570 nm (OD570). The results were expressed as the mean ± standard deviation based on triplicates. To assess the potential confounding role of antibiotics-induced reduction of bacterial density in our model, we also searched for a correlation between n-fold changes in bacterial densities and fibronectin binding levels in antibiotics-treated strain 8325-4, as compared to the untreated control.

Cell culture

All cell culture reagents were purchased from GIBCO (Paisley, UK). The human osteoblastic cell line MG-63 (LGC Standards, Teddington, UK) was grown in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine and 25 mM HEPES, 10% foetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin (culture medium) at 37°C and 5% CO_2. Cells were subcultured twice a week and used up to passage 10 after thawing.

Adhesion and invasion assay with human osteoblasts

MG-63 cells were seeded at 50,000 cells/well in 24-well plates and incubated at 37°C with 5% CO_2 for 48 h in culture medium. *S. aureus* strain 8325-4 was treated with sub-inhibitory concentrations of oxacillin, linezolid or rifampicin as described above and then washed and resuspended in antibiotic-free culture medium. The untreated *S. aureus* strain DU5883 (isogenic mutant of strain 8325-4 deleted for the genes *fnb*A/B) was used as a negative control. MG-63 cells were washed twice with DMEM and were infected with bacterial suspensions at a multiplicity of infection of approximately 50:1, as confirmed by bacterial count. Cells were incubated for 2 h at 37°C to allow for adhesion and internalisation of the bacteria and then washed twice with DMEM to remove unbound bacteria. For the adhesion assay, cells were analysed using osmotic shock in pure water and extensively pipetted to achieve full release of cell-associated bacteria. For the invasion assay, infected cells were further incubated for 1 h in culture medium containing 200 mg/L gentamicin to kill extracellular bacteria but not internalised bacteria. Cells were then washed twice in DMEM and analysed as described above to release internalised bacteria. For both the adhesion and invasion assays, viable bacteria in cell lysates were enumerated by plate counting on agar. The number of adherent bacteria was calculated by subtracting the number of internalised bacteria from the number of total cell-associated bacteria. The results were expressed as the mean ± standard deviation of the percentage of recovered internalised or adherent bacteria with respect to inoculated bacteria, derived from four independent experiments performed in duplicate.

Statistical analysis

The statistical analyses were based on the use of one-way ANOVA followed by the a posteriori Dunnett's test. Correlation analysis was performed using Spearman's rank correlation coefficient. The level of statistical significance was set at 0.05. The tests were carried out with SPSS for Windows version 12.0 software.

Results

Susceptibility to antibiotics of bacterial strains cultured in BHI

We first examined the influence of the experimental conditions on the MIC values of tested strains. The oxacillin, gentamicin, vancomycin, clindamycin, linezolid, moxifloxacin and rifampin MICs were determined using CLSI recommendations and compared to those obtained with BHI inoculated with 5×10^5 CFU/mL (Table 3). MICs in BHI were of the same magnitude as those obtained in Mueller-Hinton, therefore we used BHI medium for the rest of this study.

Effect of antibiotics on *S. aureus* adhesion to fibronectin-coated microplates

We determined the influence of antibiotics on the adhesion of *S. aureus* to fibronectin using a fibronectin-coated microplate adhesion assay. We tested standardised bacterial suspensions of 6 strains previously cultured without antibiotics or with 1/2 MIC oxacillin, gentamicin, vancomycin, clindamycin, linezolid, rifampicin or moxifloxacin. As shown in Figure 1, the adhesion to fibronectin was differentially modulated by the antibiotics. Oxacillin-, moxifloxacin-, clindamycin- and linezolid-treated bacteria displayed increased binding to fibronectin. This effect was observed for all strains tested except *fnb*A/B-negative DU5883. The increase in amplitude of fibronectin binding was strain-dependent. Oxacillin treatment increased fibronectin binding from 1.8- to 2.7-fold relative to the untreated control; moxifloxacin treatment increased binding from 1.4- to 2.3-fold; clindamycin treatment increased binding from 1.5- to 1.8-fold; and linezolid treatment increased binding from 1.6- to 2.3-fold, depending on the tested strain. By contrast, fibronectin binding was significantly reduced after rifampicin treatment. The decrease was strain-dependent and ranged from 1.5- to 3.5-fold compared to the untreated control. Vancomycin and gentamicin had no effect on bacterial adhesion to fibronectin-coated plates (data not shown). Antibiotics-induced reduction in bacterial density had no significant confounding effect on fibronectin binding in our model, as demonstrated by the absence of correlation between n-fold changes in bacterial density and fibronectin binding in antibiotics-treated strain 8325-4 (Additional File 1). The DU5883 strain, defective for *fnb*A and *fnb*B genes [9], did not adhere to fibronectin-coated plates in any condition (with or without antibiotics). Clindamycin could not be tested with the DU5883 strain as it harbours the *erm*B gene and therefore is resistant to clindamycin (Table 3).

Effect of antibiotics on fnbA and fnbB mRNA levels

We explored the effect of antibiotics on mRNA expression levels of the *fnb*A and *fnb*B genes which encode FnBPA/B. The *fnb*A and *fnb*B mRNA levels in exponential phase cultures of *S. aureus* 8325-4, grown with 1/2 MIC oxacillin, gentamicin, vancomycin, clindamycin, linezolid, rifampicin or moxifloxacin, were compared to those in an untreated culture and expressed as n-fold variation. A change in mRNA level was interpreted as significant if there was greater than 2-fold variation. As shown in Figure 2, oxacillin induced a 5.5-fold increase in the *fnb*A mRNA level and 8.5-fold increase in the *fnb*B mRNA level; moxifloxacin induced a 2.7-fold increase in the *fnb*A mRNA level and 4.5-fold increase in the *fnb*B mRNA level; and linezolid induced a 3.8-fold increase in the *fnb*A mRNA level and 6.5-fold increase in the *fnb*B mRNA level. No significant changes in fibronectin binding gene expression were observed for gentamicin, vancomycin, clindamycin or rifampicin.

Effect of antibiotics on the adhesion and invasion of osteoblastic cells

We investigated whether antibiotic-mediated modulation of the expression of *fnb*A and *fnb*B induced changes in *S. aureus* adhesion to and invasion of host cells in an *ex vivo* model. We infected osteoblastic MG-63 cells with the following: (i) *S. aureus* 8325-4, either untreated or treated with 1/2 MIC linezolid, oxacillin or rifampicin and (ii) invasion-deficient strain DU5883. We then compared the amounts of adherent and internalised bacteria recovered after 2 h. As shown in Figure 3, oxacillin-treated *S. aureus* exhibited significantly increased adhesion (682 ± 374%) compared to untreated *S. aureus* (256 ± 128%), whereas the adhesion of bacteria treated with linezolid or rifampicin (279 ± 141% and 306 ± 190%, respectively) did not differ significantly from the untreated control. Strain DU5883 showed a tendency towards impaired adhesion (151 ± 40%) compared to its parental strain 8325-4. With respect to bacterial invasion, bacteria treated with linezolid, oxacillin or rifampicin (6.7 ± 4.9%, 9.2 ± 4.1% and 10.4 ± 7.8%, respectively) did not exhibit significant differences compared to the untreated control

Table 3 MICs of antibiotics tested in BHI medium against 6 selected *S. aureus* strains

	MIC (mg/L) in BHI medium[a]					
Antibiotic	**8325-4**	**DU5883**	**ST2008 1028**	**ST2008 0563**	**HT2000 0594**	**HT2001 0390**
Oxacilllin	0.25	0.25	0.25	0.25	0.25	0.25
Gentamicin	1	1	1	1	1	1
Vancomycin	2	2	1	1	2	2
Clindamycin	0.15	> 128[b]	0.30	0.30	0.30	0.30
Linezolid	1	1	1	1	1	1
Rifampicin	0.006	0.006	0.006	0.006	0.006	0.006
Moxifloxacin	0.12	0.12	0.12	0.12	0.12	0.12

[a]MICs were determined with a standard microdilution method recommended by the CSLI in BHI medium inoculated with 5×10^5 CFU/mL [b]DU5883 strain is resistant to clindamycin as it harbours the *erm*B gene [9]

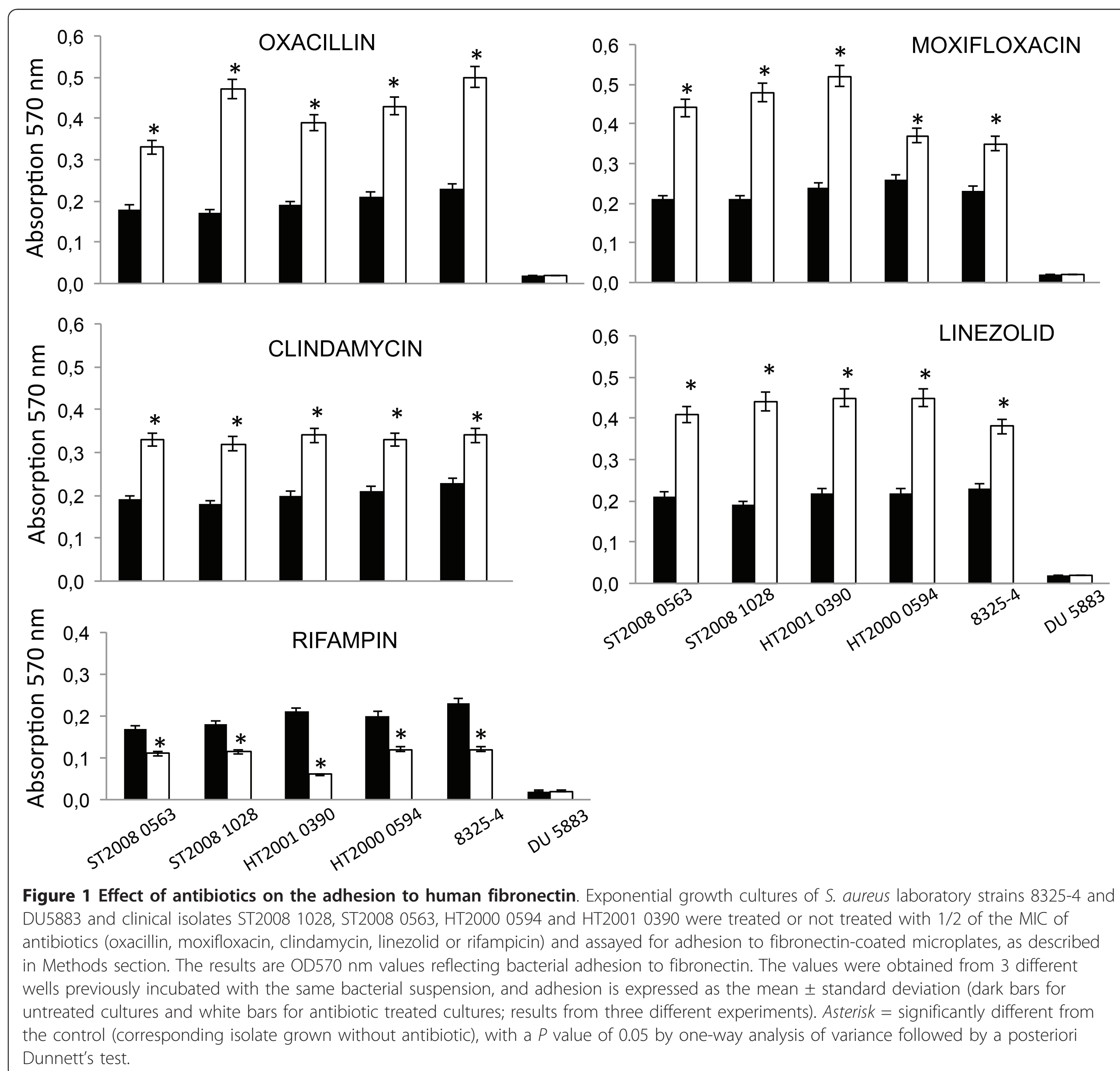

Figure 1 Effect of antibiotics on the adhesion to human fibronectin. Exponential growth cultures of *S. aureus* laboratory strains 8325-4 and DU5883 and clinical isolates ST2008 1028, ST2008 0563, HT2000 0594 and HT2001 0390 were treated or not treated with 1/2 of the MIC of antibiotics (oxacillin, moxifloxacin, clindamycin, linezolid or rifampicin) and assayed for adhesion to fibronectin-coated microplates, as described in Methods section. The results are OD570 nm values reflecting bacterial adhesion to fibronectin. The values were obtained from 3 different wells previously incubated with the same bacterial suspension, and adhesion is expressed as the mean ± standard deviation (dark bars for untreated cultures and white bars for antibiotic treated cultures; results from three different experiments). *Asterisk* = significantly different from the control (corresponding isolate grown without antibiotic), with a *P* value of 0.05 by one-way analysis of variance followed by a posteriori Dunnett's test.

(6.0 ± 5.1%), while host cell invasion was abolished in strain DU5883 lacking *fnb*A and *fnb*B (0.0 ± 0.0%).

Discussion

Several major findings emerge from this investigation of the impact of sub-inhibitory concentrations of anti-staphylococcal drugs on *S. aureus* adhesion and invasion phenotypes. *S. aureus* binding to human fibronectin and the transcriptional levels of the *fnb*A/B genes encoding the fibronectin-binding proteins were differentially modulated by antimicrobial agents. Oxacillin, moxifloxacin and linezolid treatment led to the development of a hyper-adhesive phenotype, along with an increase in *fnb*A/B mRNA levels relative to the *gyr*B internal standard. The same hyper-adhesive phenotype was induced by clindamycin treatment, although no significant change in *fnb*A/B mRNA levels was observed. Rifampin was the only antimicrobial agent among those tested that significantly inhibited *S. aureus* binding to fibronectin without affecting relative *fnb*A/B transcription profiles. Vancomycin and gentamicin induced no change in either the adhesion phenotype or the *fnb*A/B transcription. *S. aureus* adhesion to and invasion of live eukaryotic cells was also assessed after oxacillin, linezolid or rifampin treatment in an *ex vivo* infection model of cultured human osteoblasts. Oxacillin treatment significantly increased *S. aureus* adhesion but

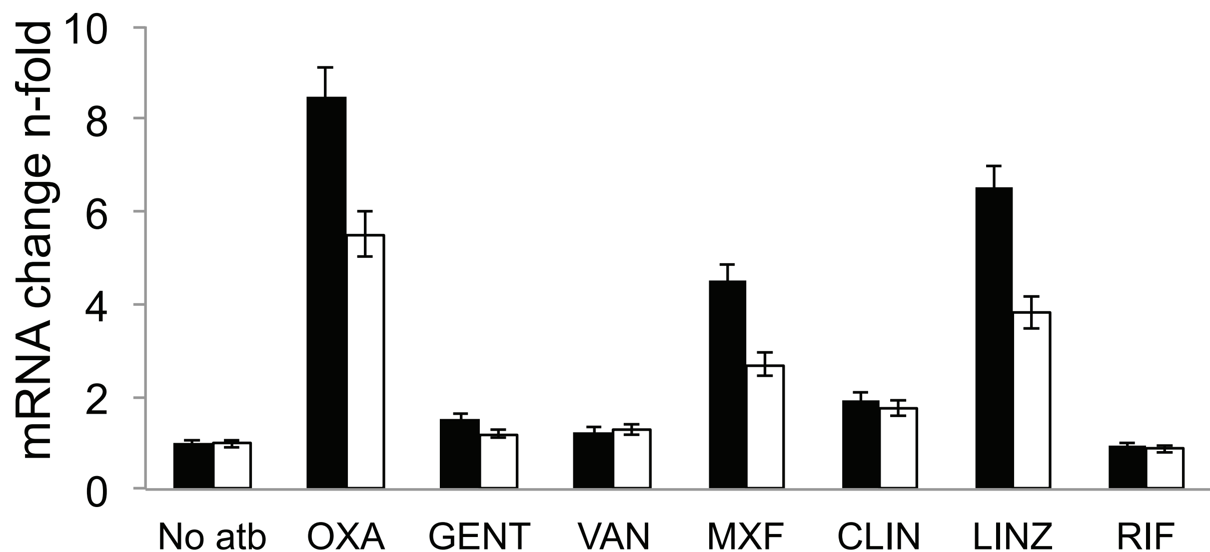

Figure 2 Effect of antibiotics on *fnb*A and *fnb*B mRNA levels. Exponentially growing cultures of *S. aureus* 8325-4 were treated for 2 h with no antibiotics or with 1/2 the MIC of oxacillin, gentamicin, vancomycin, moxifloxacin, clindamycin, linezolid or rifampicin. Samples of each culture were taken and adjusted to an OD600 of 1 and then used for total RNA extraction and subsequent reverse transcription with random primers, as described above. The cDNA obtained was used as the template for LightCycler PCR with specific *fnb*A, *fnb*B and *gyr*B primers. Relative quantification was performed by reporting it relative to *gyr*B expression, as described elsewhere [14]. The results are expressed as the n-fold variation of *fnb*A (*white bars*) and *fnb*B (*black bars*) mRNA levels in the presence of each antibiotic relative to the growth of no antibiotic control levels. The values are the means ± standard deviations (four different experiments). A change in mRNA level was interpreted as significant if greater than 2-fold variation.

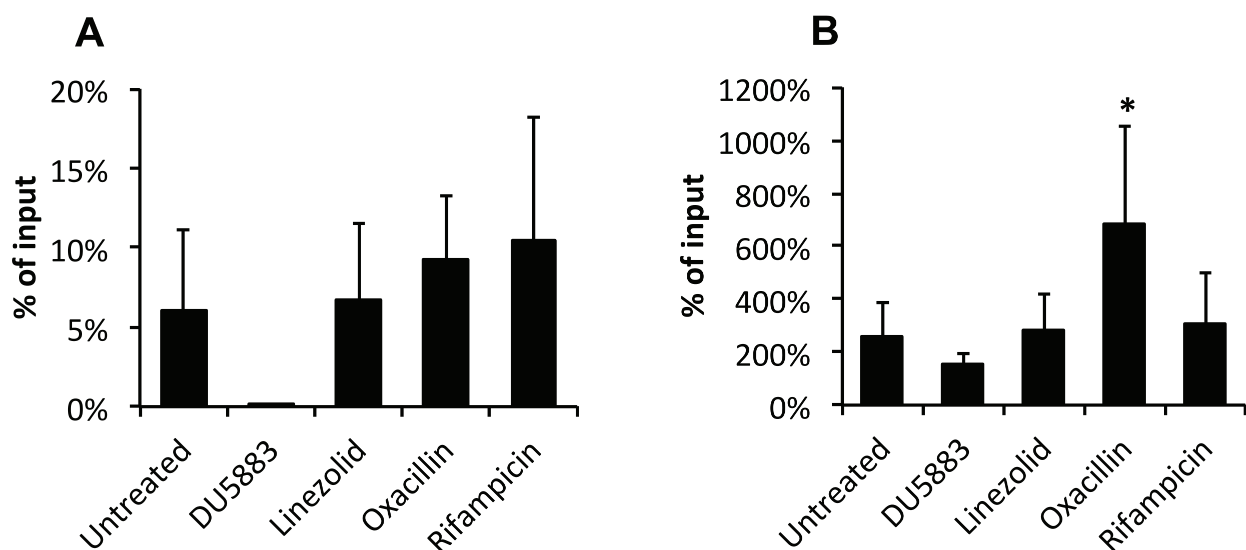

Figure 3 Effect of antibiotics on *S. aureus* adhesion to and invasion of human osteoblasts. MG-63 osteoblastic cells were infected for 2 h at approximately 50 bacteria/cell with *S. aureus* strain 8325-4, pre-treated or not (untreated control) with 1/2 MIC linezolid, oxacillin or rifampicin, and *S. aureus* strain DU5883 lacking *fnb*A and *fnb*B (negative control). To enumerate cell-associated bacteria, infected cells were washed twice to discard unbound bacteria and analysed by osmotic shock in pure water, and then, suitable dilutions of the lysates were plated on agar. The same procedure was used to quantify intracellular bacteria, except that the cells were incubated for 1 h with 200 mg/L gentamicin before the lysis step to kill extracellular bacteria. Adherent bacteria were calculated by subtracting intracellular bacteria from cell-associated bacteria. The results were expressed as the means +/- standard deviation of the percentage of recovered internalised (**a**) or adherent (**b**) bacteria with respect to inoculated bacteria derived from four independent experiments performed in duplicate. *Asterisk* = significantly different from the control (corresponding isolate grown without antibiotic), with a *P* value of 0.05 by one-way analysis of variance followed by a posteriori Dunnett's test.

not invasion, while no significant change in adhesion or invasion levels was observed after linezolid or rifampin treatment.

Several recent studies have focused on the influences of sub-inhibitory concentrations of antimicrobial agents on the expression of various virulence factors produced by *S. aureus* and on the various regulation mechanisms involved in this modulation [6,8,17]. Similarly, the expression and surface display of FnBPs are regulated by a complex network of global regulators and stress response pathways that can be triggered by antimicrobial agents in a drug-specific fashion. Fluoroquinolones, by activating the SOS system (a global response system to DNA damage), have been shown to induce *fnb*B up-regulation and fibronectin binding in *S. aureus* through a LexA-RecA-dependant pathway [18]. Moreover, in a rabbit *S. aureus* infection model, moxifloxacin treatment inhibited the expression of *agr* global regulator [19], which acts as a repressor of surface protein expression, including *fnb*A/B, and as an activator of exotoxin expression [20]. Beta-lactams, besides inducing the SOS response system [21], have also been reported to up-regulate virulence factor expression, including *fnb*B, through the two-component system SaeRS [22].

Clindamycin and linezolid are protein synthesis inhibitory agents known to repress exotoxin secretion by *S. aureus* [6-8]. Thus, their positive effect on fibronectin binding in *S. aureus* makes it tempting to speculate that their impact on protein expression involves selective inhibition of *agr*. We recently showed that sub-inhibitory concentrations of linezolid repress early *agr* expression in *S. aureus* [23]. Furthermore, sub-inhibitory concentrations of clindamycin have been shown to decrease *sae*RS expression [24], thus possibly interfering with *fnb*B expression. An alternative explanation for the effects of clindamycin has been reported by Blickwede et al., who observed that *fnb*B mRNA levels were selectively increased after clindamycin treatment and that this increase was due to mRNA stabilisation in the presence of clindamycin [25]. Whether linezolid also affects fnbA/B mRNA levels through mRNA stabilisation remains unknown, and this question merits further investigations.

With respect to sub-inhibitory rifampin treatment, the decrease in fibronectin binding observed here was not accompanied by a transcriptional decrease of *fnb*A/B relative to the internal control *gyr*B, suggesting that fibronectin binding inhibition takes place at the post-transcriptional level. Mechanisms underlying the effects of rifampin in this context are still to be elucidated. We speculate that these mechanisms could involve either a decrease of sortase activity, which is responsible for cell wall anchorage of several MSCRAMMs including FnBPs [26,27], or an increase of protease activity, which has been shown to dramatically influence fibronectin-binding in *S. aureus* [28].

Interestingly, fibronectin-binding modulation by oxacillin, linezolid or rifampin only partially correlated with host cell adhesion and invasion under our experimental conditions. Although oxacillin-treated *S. aureus* displayed significantly increased binding to cultured osteoblasts, its invasiveness did not differ significantly from that of the untreated control. Beta-lactams interfere with cell division and induce dramatic changes in staphylococcal morphology even at sub-inhibitory concentrations [29]. Of note, the inhibition of cell separation ultimately leads to the formation of so-called pseudomulticellular staphylococci [30]. These aberrant forms were present following oxacillin treatment under our experimental conditions, whereas bacterial size and morphology were unchanged in bacteria either untreated or treated with rifampin or linezolid, as objectivated by microscopic examination after fluorescence staining of the cell wall (data not shown). It is likely that the larger size of pseudomulticellular staphylococci hampers their internalization by osteoblasts, which could negatively compensate the increase in adhesiveness induced by oxacillin. In the same way, we failed to identify a change in adhesion and invasion phenotypes after linezolid or rifampin treatment. A putative explanation for these discrepancies between phenotypes observed under both controlled *in vitro* conditions and more complex *ex vivo* infection assays is adhesin redundancy. Although FnBPs play a major role in *S. aureus*-host cell interactions, whole cell adhesion involves several other MSCRAMMs [31], which are also likely regulated by antibiotics and thus could hamper or cancel the effects of FnBPs modulation. This outcome is illustrated by our finding that strain DU5883 lacking *fnb*A/B still adhered significantly to cultured osteoblasts. The same is probably true with respect to *S. aureus* invasiveness, although a more limited number of factors are involved along with FnBPs in the cell invasion process. FnBPs are required and sufficient for host cell invasion [27], as confirmed in our model by the observation that invasiveness was abolished in strain DU5883. However, the multifunctional protein *eap*, which also binds fibronectin, acts additively with FnBPs to mediate host cell invasion in *eap*-positive strains such as 8325-4 [32] and can partially compensate for loss of FnBP functions [27]. Additional studies are warranted to determine whether compensatory mechanisms occur to sustain host cell invasion, despite rifampin-mediated FnBP expression decrease.

Conclusions

It has long been well-established that the choice of antimicrobial agents in therapy should not solely rely on their respective bactericidal or bacteriostatic activity and

pharmacokinetics but should also take into account their influence on bacterial virulence [33,34], including adhesion phenotype. Our results confirm that several anti-staphylococcal agents induce a hyper-adhesive phenotype in *S. aureus* through FnBP up-regulation *in vitro*, while only rifampin inhibits fibronectin binding. However, drug-dependent modulation of adhesion, although unambiguous at the molecular and specific ligand-binding level, was not always significant in our *ex vivo* model. This paradoxical observation is reminiscent of that recently reported by Ythier et al., who demonstrated that *in vitro* adherence to fibronectin of clinical *S. aureus* isolates did not correlate with infectivity in a rat model of endocarditis [35]. While antibiotic-mediated modulation of secreted exotoxins has proved fully relevant in the clinical field with respect to several toxin-associated diseases [33,34], the relevance of adhesion modulation during *in vivo* infections is still uncertain and requires further investigation.

Additional material

Additional file 1: Impact of antibiotics on the growth kinetics of S. aureus strain 8325-4 and correlation analysis between n-fold changes in bacterial density and fibronectin binding. Panel A. Bacterial suspensions were cultivated with or without antibiotics at half-MIC for 2 h as described above. Growth curves with and without antibiotics are represented as Δ log variations of the bacterial density. Panel B. Antibiotics-treated suspensions of S. aureus 8325-4 were assayed for fibronectin binding as described above. Spearman's rank correlation coefficient was calculated and no correlation was found between the bacterial density changes and fibronectin binding measures.

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Author details
[1]INSERM, U851, Lyon 69008, France. [2]Centre National de référence des Staphylocoques, Faculté de Médecine Lyon Est, Université de Lyon, Lyon 69008, France. [3]Centre National de Référence des Staphylocoques, 7 rue Guillaume Paradin, 69372 Lyon cedex 08, France.

Authors' contributions
JPR, YL carried out the *ex vivo* adhesion and invasion assays. AM, OD carried out the adhesion and RT-PCR assays. JPR and OD drafted the manuscript. GL, AT, MB participated in the design of the study and performed the statistical analysis. GL, FL, FV, JE conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1. Foster TJ, Hook M: **Surface protein adhesins of *Staphylococcus aureus*.** *Trends Microbiol* 1998, **6(12)**:484-488.
2. Sinha B, Francois P, Que YA, Hussain M, Heilmann C, Moreillon P, Lew D, Krause KH, Peters G, Herrmann M: **Heterologously expressed *Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells.** *Infection and immunity* 2000, **68(12)**:6871-6878.
3. Ahmed S, Meghji S, Williams RJ, Henderson B, Brock JH, Nair SP: ***Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria.** *Infect Immun* 2001, **69(5)**:2872-2877.
4. Stevens QE, Seibly JM, Chen YH, Dickerman RD, Noel J, Kattner KA: **Reactivation of dormant lumbar methicillin-resistant *Staphylococcus aureus* osteomyelitis after 12 years.** *J Clin Neurosci* 2007, **14(6)**:585-589.
5. Stevens DL, Ma Y, Salmi DB, McIndoo E, Wallace RJ, Bryant AE: **Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*.** *J Infect Dis* 2007, **195(2)**:202-211.
6. Herbert S, Barry P, Novick RP: **Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*.** *Infect Immun* 2001, **69(5)**:2996-3003.
7. Bernardo K, Pakulat N, Fleer S, Schnaith A, Utermohlen O, Krut O, Muller S, Kronke M: **Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression.** *Antimicrob Agents Chemother* 2004, **48(2)**:546-555.
8. Dumitrescu O, Boisset S, Badiou C, Bes M, Benito Y, Reverdy ME, Vandenesch F, Etienne J, Lina G: **Effect of antibiotics on *Staphylococcus aureus* producing Panton-Valentine leukocidin.** *Antimicrob Agents Chemother* 2007, **51(4)**:1515-1519.
9. Greene C, McDevitt D, Francois P, Vaudaux PE, Lew DP, Foster TJ: **Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes.** *Mol Microbiol* 1995, **17(6)**:1143-1152.
10. Tristan A, Ying L, Bes M, Etienne J, Vandenesch F, Lina G: **Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections.** *J Clin Microbiol* 2003, **41(9)**:4465-4467.
11. Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F: **Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease.** *Infect Immun* 2002, **70(2)**:631-641.
12. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG: **Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*.** *J Clin Microbiol* 2000, **38(3)**:1008-1015.
13. National Committee for Clinical Laboratory Standards: **Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard.** NCCLS document M7-A7. Wayne (PA): NCCLS;, 7 2006.
14. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, Benito Y, Barbu EM, Vazquez V, Hook M, Etienne J, *et al*: ***Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia.** *Science* 2007, **315(5815)**:1130-1133.
15. Goerke C, Fluckiger U, Steinhuber A, Bisanzio V, Ulrich M, Bischoff M, Patti JM, Wolz C: **Role of *Staphylococcus aureus* global regulators *sae* and *sigma*B in virulence gene expression during device-related infection.** *Infect Immun* 2005, **73(6)**:3415-3421.
16. Vaudaux P, Francois P, Bisognano C, Kelley WL, Lew DP, Schrenzel J, Proctor RA, McNamara PJ, Peters G, Von Eiff C: **Increased expression of clumping factor and fibronectin-binding proteins by *hem*B mutants of *Staphylococcus aureus* expressing small colony variant phenotypes.** *Infect Immun* 2002, **70(10)**:5428-5437.
17. Dickgiesser N, Wallach U: **Toxic shock syndrome toxin-1 (TSST-1): influence of its production by subinhibitory antibiotic concentrations.** *Infection* 1987, **15(5)**:351-353.
18. Bisognano C, Kelley WL, Estoppey T, Francois P, Schrenzel J, Li D, Lew DP, Hooper DC, Cheung AL, Vaudaux P: **A RecA-LexA-dependent pathway mediates ciprofloxacin-induced fibronectin binding in *Staphylococcus aureus*.** *J Biol Chem* 2004, **279(10)**:9064-9071.
19. Bronner S, Jehl F, Peter JD, Ploy MC, Renault C, Arvis P, Monteil H, Prevost G: **Moxifloxacin efficacy and vitreous penetration in a rabbit model of *Staphylococcus aureus* endophthalmitis and effect on gene expression of leucotoxins and virulence regulator factors.** *Antimicrob Agents Chemother* 2003, **47(5)**:1621-1629.
20. Novick RP: **Autoinduction and signal transduction in the regulation of staphylococcal virulence.** *Mol Microbiol* 2003, **48(6)**:1429-1449.

21. Maiques E, Ubeda C, Campoy S, Salvador N, Lasa I, Novick RP, Barbe J, Penades JR: **Beta-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in *Staphylococcus aureus*.** *J Bacteriol* 2006, **188(7)**:2726-2729.
22. Kuroda H, Kuroda M, Cui L, Hiramatsu K: **Subinhibitory concentrations of beta-lactam induce haemolytic activity in *Staphylococcus aureus* through the *Sae*RS two-component system.** *FEMS Microbiol Lett* 2007, **268(1)**:98-105.
23. Dumitrescu O, Forey F, Bes M, Vandenesch F, Etienne J, Lina G: **Linezolid decreases exotoxins expression in *Staphylococcus aureus* by early repressing *agr*, *sar*A and *sae* regulators.** *21st ECCMID and the 27th ICC* 2011.
24. Novick RP, Jiang D: **The staphylococcal *sae*RS system coordinates environmental signals with *agr* quorum sensing.** *Microbiology* 2003, **149(Pt 10)**:2709-2717.
25. Blickwede M, Wolz C, Valentin-Weigand P, Schwarz S: **Influence of clindamycin on the stability of *coa* and *fnb*B transcripts and adherence properties of *Staphylococcus aureus* Newman.** *FEMS Microbiol Lett* 2005, **252(1)**:73-78.
26. Grundmeier M, Hussain M, Becker P, Heilmann C, Peters G, Sinha B: **Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function.** *Infect Immun* 2004, **72(12)**:7155-7163.
27. Sinha B, Fraunholz M: ***Staphylococcus aureus* host cell invasion and post-invasion events.** *Int J Med Microbiol* 2010, **300(2-3)**:170-175.
28. McGavin MJ, Zahradka C, Rice K, Scott JE: **Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease.** *Infect Immun* 1997, **65(7)**:2621-2628.
29. Lorian V: **Some effect of subinbilitory concentrations of penicillin on the structure and division of staphylococci.** *Antimicrob Agents Chemother* 1975, **7(6)**:864-867.
30. Giesbrecht P, Kersten T, Maidhof H, Wecke J: **Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin.** *Microbiol Mol Biol Rev* 1998, **62(4)**:1371-1414.
31. Hauck CR, Ohlsen K: **Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*.** *Curr Opin Microbiol* 2006, **9(1)**:5-11.
32. Harraghy N, Kormanec J, Wolz C, Homerova D, Goerke C, Ohlsen K, Qazi S, Hill P, Herrmann M: ***sae* is essential for expression of the staphylococcal adhesins Eap and Emp.** *Microbiology* 2005, **151(Pt 6)**:1789-1800.
33. Lappin E, Ferguson AJ: **Gram-positive toxic shock syndromes.** *Lancet Infect Dis* 2009, **9(5)**:281-290.
34. Micek ST, Dunne M, Kollef MH: **Pleuropulmonary complications of Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus*: importance of treatment with antimicrobials inhibiting exotoxin production.** *Chest* 2005, **128(4)**:2732-2738.
35. Ythier M, Entenza JM, Bille J, Vandenesch F, Bes M, Moreillon P, Sakwinska O: **Natural variability of in vitro adherence to fibrinogen and fibronectin does not correlate with in vivo infectivity of *Staphylococcus aureus*.** *Infect Immun* 2010, **78(4)**:1711-1716.

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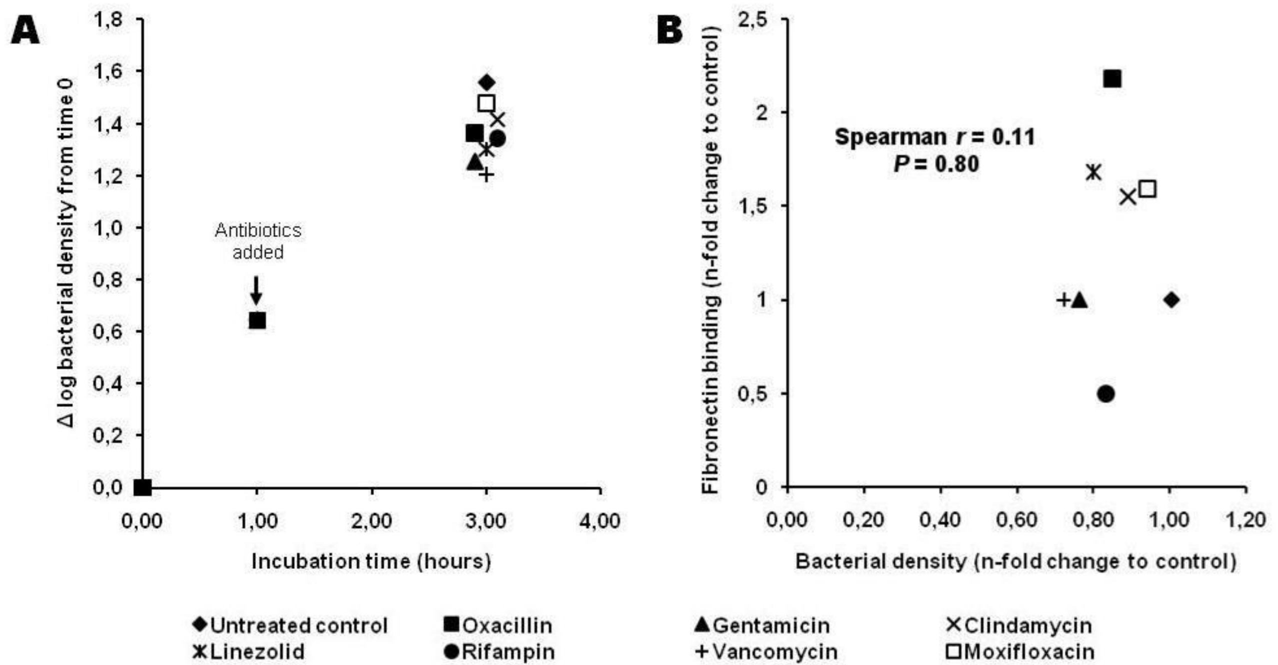

Additional File 1. Impact of antibiotics on the growth kinetics of *S. aureus* strain 8325-4 and correlation analysis between n-fold changes in bacterial density and fibronectin binding. Panel A. Bacterial suspensions were cultivated for 1 h then treated with half-MIC antibiotics for 2 h. Bacterial density was measured at 0, 1 and 3 h using McFarland turbidity scale. Results were expressed as Δ log variations to bacterial density measured at time 0. **Panel B.** Antibiotics-treated suspensions of *S. aureus* 8325-4 were tested for fibronectin binding using a fibronectin-coated microplate assay and spectrophotometric quantification as described in Methods. Spearman's rank correlation coefficient was calculated for n-fold changes in bacterial density and fibronectin binding as compared to the untreated control. No correlation was found between these two variables, thus indicating that antibiotics-induced reduction in bacterial density had no significant confounding effect in our model.

V. DISCUSSION

Nos travaux de recherche ont abordé les interactions entre *S. aureus* et les ostéoblastes tout d'abord sous un angle méthodologique, puis sous l'angle de la variabilité intergénotypique de ces interactions et de leur concordance avec la présentation clinique des IOA à CA-MRSA et HA-MRSA, et enfin sous l'angle de l'influence exercée par les traitements antibiotiques sur ces interactions.

La mise au point d'une méthode d'étude par cytométrie en flux de l'adhésion et de l'invasion bactérienne a comblé un manque dans l'arsenal méthodologique disponible ; cette méthode a pu être utilisée avec succès au cours de différents projets de notre équipe, sans rapports directs avec les travaux présentés dans cette thèse. Cependant, différentes limites de cette approche, qui ont été soulignées dans la discussion de l'article publié, l'ont rendue incompatible avec la suite des travaux personnels présentés dans cette thèse. La première limite a trait au choix du composé bactéricide utilisé pour la destruction des bactéries extracellulaires. L'approche choisie emploie la lysostaphine dans ce but ; lorsque nous avons utilisé la cytométrie en flux pour comparer les pouvoirs invasifs de différents MRSA, il s'est avéré que certaines souches présentaient une sensibilité diminuée à ce composé. En conséquence, la destruction des bactéries adhérentes pouvait être incomplète, aboutissant à une fluorescence résiduelle à l'origine d'erreurs de mesure. Nous avons conclu que l'emploi de la cytométrie en flux avec sélection à la lysostaphine devait être réservé à l'étude de souches non-MRSA. Une autre limite technique nous a empêché d'utiliser cette approche dans nos travaux sur l'impact des antibiotiques : l'application d'antibiotiques à doses sub-inhibitrices, particulièrement les β-lactamines, induit des modifications morphologiques majeures chez *S. aureus* qui introduisent des distorsions entre le niveau de marquage fluorescent et la charge bactérienne réelle. Enfin, le choix de la cytométrie en flux avait pour objectif initial de s'affranchir du caractère viable ou non des bactéries quantifiées. Dans nos travaux portant sur les MRSA, nous avons cherché à privilégier la pertinence clinique des modèles d'infection utilisés et à quantifier la survie bactérienne plutôt que l'invasion

pour elle-même ; en conséquence, les mesures d'internalisation devaient tenir compte de la viabilité des bactéries. Comme décrit dans la seconde publication, nous avons alors fait le choix de modifier et optimiser la technique classique de protection à la gentamicine, d'une part en automatisant les étapes d'ensemencement et de numération des colonies, d'autre part en ayant recours à la sonication des suspensions bactériennes pour améliorer la précision des mesures. Cette approche s'est avérée satisfaisante en termes de débit, de robustesse et de précision, et a été privilégiée sur la cytométrie dans la suite de nos travaux.

Nos résultats sur l'invasion des ostéoblastes par les MRSA ont mis en lumière un nouveau trait de virulence des CA-MRSA. La destruction des cellules osseuses par ces pathogènes permet d'ébaucher un nouveau modèle physiopathologique de l'IOA à CA-MRSA : dans ce modèle candidat, la destruction osseuse résulte non seulement de l'action indirecte de la PVL, via la recrutement et la lyse des leucocytes au site de l'infection, mais également de l'action directe des PSM sur les ostéoblastes envahis. Ces observations en modèle ex vivo, bien que concordantes avec la gravité clinique des IOA à CA-MRSA, soulèvent plusieurs questions.

La première question est celle de l'action proprement dite des PSM. Les données de la littérature suggèrent que les PSM sont effectivement sécrétés à l'intérieur des phagosomes à l'issue de l'étape d'invasion (Surewaard *et al.*, 2012) ; de plus, l'expression des PSM est sous le contrôle d'*agr* (Thoendel *et al.*, 2011). Ce dernier est activé lors de l'internalisation dans le phagosome (Qazi *et al.*, 2001). Dans un milieu libre, l'activation du système *agr* est dépendante de la concentration en peptide auto-inducteur, concentration elle-même dépendante de la concentration bactérienne. *agr* fonctionne donc dans ce contexte comme un système de quorum sensing. Cependant, dans le compartiment phagosomal, ce n'est plus la concentration bactérienne qui aboutit à l'accumulation du peptide auto-inducteur mais le caractère confiné de la vacuole. Dans ce contexte intracellulaire, *agr* se comporte donc comme un détecteur de confinement et déclenche ainsi la sécrétion des exotoxines nécessaires à l'échappement phagosomal (Qazi *et al.*, 2001). Ces observations ont été réalisées avant la découverte des PSM (Wang *et al.*, 2007). Nos données sur le rôle des PSM dans la cytotoxicité, à la lumière de la dépendance de ces toxines vis-à-vis d'*agr*, confirment ainsi le modèle proposé par Qazi et al. dans

lequel l’activation d’*agr* par confinement est un pré-requis à l’échappement phagosomal. Cependant, la notion même d’échappement phagosomal comme préalable à l’expression de la cytotoxicité a récemment été remise en question lorsque Kobayashi *et al*. ont observé que les CA-MRSA USA300 phagocytés par des polynucléaires neutrophiles pouvaient provoquer la mort de ces derniers sans échappement phagosomal (Kobayashi *et al.*, 2010). Il est utile de souligner qu’un rôle des PSM dans ce phénotype n’avait pas été recherché. Bien que les polynucléaires et les ostéoblastes soient des types cellulaires très différents, il est possible que l’expression de PSM chez les ostéoblastes puisse provoquer leur mort sans destruction du phagosome. Un modèle explicatif candidat pourait être que la perméabilisation de la membrane phagosomale par les PSM, éventuellement en synergie avec d’autres PFT, soit suffisante pour permettre le relargage de PSM dans le cytoplasme de la cellule hôte et l’induction de sa mort sans nécessiter l’échappement phagosomal de la bactérie elle-même. De plus, à la lumière du caractère non spécifique de l’action des PSM, il semble important d’explorer leur action chez d’autres types cellulaires comme les polynucléaires neutrophiles et les cellules endothéliales.

La surexpression des PSM par les CA-MRSA s’impose désormais comme un déterminant majeur de leur virulence (Wang *et al.*, 2007; Otto, 2010). Cette surexpression semble associée à un niveau d’expression élevé d’*agr* chez ces souches par comparaison avec d’autres fonds génétiques (Montgomery *et al.*, 2010). Ces observations ont fait émerger une question majeure dans l’étude de la pathogénie des CA-MRSA, qui est celle de l’origine de cette surexpression des régulateurs de virulence. Des investigations en ce sens sont donc nécessaires, et leur mise en place dans le cadre d’un projet spécifique sera discutée au prochain chapitre.

Bien que la démonstration du rôle des PSM dans la mort des ostéoblastes infectés apporte un gain de connaissance sur la pathogénie des CA-MRSA, une question reste en suspens : quelles sont les contributions relatives de la PVL et des PSM dans la destruction tissulaire au cours des IOA à CA-MRSA ? En effet, nos résultats ont été obtenus ex vivo, dans des conditions contrôlées où n’entrent pas en compte l’action indirecte de la PVL via le système immunitaire. L’exploration de ces

contributions relatives s'impose donc comme un pré-requis à la recherche de stratégies thérapeutiques optimisées ciblant la synthèse des PSM ; cependant, il est d'ores et déjà envisageable que l'emploi d'antibiotiques à visée anti-toxinique comme la clindamycine, en atténuant la production de PVL (Dumitrescu *et al.*, 2007), atténue aussi celle des PSM. D'une part, les voies de régulation de la PVL et des PSM sont interdépendantes via *agr* (Thoendel *et al.*, 2011), et d'autre part les antibiotiques à visée anti-toxinique ont montré des actions analogues sur différentes exotoxines comme la PVL, l'α-toxine et les superantigènes (Herbert *et al.*, 2001; Bernardo *et al.*, 2004; Stevens *et al.*, 2007). Il est donc probable que l'expression des PSM soit atténuée par ces antibiotiques comme celle des autres exotoxines.

L'hypothèse d'un rôle physiopathologique de l'internalisation de *S. aureus* par les ostéoblastes est largement acceptée depuis plusieurs années (Ellington *et al.*, 2003; Ellington *et al.*, 2006). Nos observations sur la capacité de survie intracellulaire des HA-MRSA suggèrent que la constitution d'un réservoir bactérien intracellulaire est associée à la chronicité des IOA. Il est tout à fait notable que les HA-MRSA, classiquement associés à des formes d'infections osseuses plus chroniques ou indolentes que les CA-MRSA, présentent une capacité d'invasion et de survie intracellulaire supérieure à ces derniers. Ces résultats n'apportent cependant qu'un argument indirect en faveur du rôle de l'internalisation dans la chronicité. Il reste nécessaire, pour confirmer définitivement cette hypothèse, de confronter des données cliniques et microbiologiques issues d'une même étude pour rechercher une association entre chronicité des IOA et internalisation de *S. aureus*. La mise en place d'une telle étude sera discutée dans le chapitre suivant.

Nous avons observé que la délétion du gène de l'α-toxine chez deux souches USA300 était associée à un gain de cytotoxicité. Tout se passe donc comme si l'α-toxine jouait un rôle protecteur pour les ostéoblastes vis-à-vis de la mort induite par les *S. aureus* intracellulaires. Cette observation est, en première analyse, surprenante à plus d'un titre : l'α-toxine est considérée comme une toxine cytotoxique majeure de *S. aureus* et a été impliquée dans l'échappement phagosomal et l'induction de l'apoptose chez différents types cellulaires (Walev *et al.*, 1993; Bantel *et al.*, 2001; Haslinger *et al.*, 2003; Haslinger-Loffler *et al.*, 2005; Nygaard *et al.*, 2012). Cependant, certaines données de la littérature, combinées à

nos observations sur le rôle des PSM dans la cytotoxicité de *S. aureus*, permettent de proposer un modèle compatible avec un rôle non cytotoxique de l'α-toxine vis-à-vis des ostéoblastes. Premièrement, il a été observé que l'α-toxine pouvait fixer l'intégrine β-1 de la cellule hôte et entrer en compétition avec la fixation de la fibronectine, interférant de ce fait avec l'adhésion et l'internalisation bactérienne chez les cellules respiratoires A549 (Liang et Ji, 2006). Il est donc envisageable qu'une réduction dans le nombre de bactéries internalisées imputable à la sécrétion d'α-toxine soit indirectement responsable d'une diminution des dommages subis par les cellules hôtes. Cependant, l'impact de la délétion du gène *hla* sur la capacité d'invasion de *S. aureus* n'a pas pu être mise en évidence dans notre modèle. Les conditions de l'étape d'infection sont probablement incompatibles avec l'observation de ce phénotype : l'étape de coculture entre bactéries et ostéoblastes étant limitée à deux heures, il est peu probable que cette durée permette une accumulation d'α-toxine suffisante pour exercer un impact mesurable en termes d'invasion. Une explication plus plausible pour le rôle protecteur de l'α-toxine repose sur les résultats de Giese et al (Giese *et al.*, 2011). Ces auteurs ont montré qu'une synergie entre δ-toxine et β-toxine était nécessaire à l'échappement phagosomal chez la souche *S. aureus* RN4220. Ces résultats sont difficilement extrapolables aux souches cliniques de *S. aureus*, notamment aux CA-MRSA, car ces derniers n'expriment pas de β-toxine fonctionnelle, le gène *hlb* étant inactivé par l'insertion d'un phage dans le cadre de lecture (Carroll *et al.*, 1993). Cependant, ils fournissent une hypothèse plausible dans laquelle l'α-toxine interagirait négativement avec l'échappement ou la perméabilisation phagosomale en inhibant l'activité d'une autre toxine nécessaire à cet échappement ou à cette perméabilisation. En effet, l'existence d'une interaction négative entre α-toxine et β-toxine est clairement documentée (Herbert *et al.*, 2010), et il est envisageable qu'une telle interférence ait lieu entre l'α-toxine et d'autres toxines cytotoxiques des CA-MRSA impliquées dans la perméabilisation du phagosome.

Nos investigations sur l'impact des antibiotiques à doses sub-inhibitrices sur l'adhésion et l'invasion de *S. aureus* avaient pour objectif de vérifier que certains antibiotiques utilisés dans la prophylaxie et le traitement des IOA comme les β-lactamines n'induisaient pas un surcroît de capacité d'invasion. Nous avons pu montrer in vitro que cette hypothèse était fondée, et qu'une modulation

transcriptionnelle positive de *fnb*A/B était exercée par l'oxacilline, la moxifloxacine et le linézolide. Cette modulation se traduisait par un gain d'adhésion à la fibronectine ; la clindamycine était également associée à un gain d'adhésion à la fibronectine, et la rifampicine à une perte d'adhésion, d'origine probablement post-transcriptionnelle puisque que ces antibiotiques n'induisaient pas de modification mesurable des niveaux d'ARNm de *fnb*A et *fnb*B. Cependant, la seule modification phénotypique induite par les antibiotiques lors de mesures ex vivo d'adhésion et d'invasion de *S. aureus* sur ostéoblastes était un gain d'adhésion associé à l'oxacilline. En particulier, aucune perte d'adhésion ou d'invasion n'a été observée en présence de rifampicine, malgré des résultats in vitro montrant une perte de fixation à la fibronectine. Bien que les FnBP soient des acteurs majeurs dans les phénomènes d'adhésion et d'invasion cellulaire, le caractère redondant des adhésines de *S. aureus* est probablement à l'origine de ces discordances en modèle in vitro et ex vivo. En effet, les effets mesurés in vitro lors des études de liaison à la fibronectine présentaient une amplitude d'un facteur entre 1.5 et 2.0. En particulier, la diminution de l'adhésion à la fibronectine induite par la rifampicine était d'amplitude nettement plus faible que celle induite par la délétion des gènes *fnb*A/B chez la souche DU5883. En conséquence, il est probable que cette amplitude d'effet partielle soit compensée, ex vivo et très probablement in vivo, par la présence d'autres adhésines redondantes comme Eap (Harraghy *et al.*, 2005; Hussain *et al.*, 2008). Comme précisé dans la discussion de l'article publié, il a été observé précédemment que l'adhésion à la fibronectine in vitro n'était pas corrélé à l'infectivité de *S. aureus* dans un modèle d'endocardite expérimentale chez le rat (Ythier *et al.*, 2010). Au final, nous considérons que bien que les données in vitro indiquent un effet de certains antibiotiques sur l'adhésion à la fibronectine de *S. aureus*, les données ex vivo ne permettent pas de conclure à une pertinence clinique potentielle de ces observations.

VI. PERSPECTIVES

VI.A. Pertinence clinique de l'internalisation de *S. aureus* par les ostéoblastes au cours des IOA

Nous avons établi que les HA-MRSA, associés à des IOA fréquemment chroniques, présentent une capacité d'invasion et de survie intracellulaire dans les ostéoblastes supérieure à celle des CA-MRSA, associés à des IOA aiguës. Ces résultats sont en faveur d'une association in vivo entre internalisation et chronicité, mais la preuve définitive de cette association n'a jamais été apportée. Nous travaillons actuellement en ce sens, dans le cadre d'une étude conjointe clinico-biologique. L'objectif de cette étude est d'établir une corrélation statistique entre le délai d'évolution de l'IOA et la capacité d'invasion de la souche de *S. aureus* responsable. Notre approche repose sur la constitution d'une cohorte de 100 patients atteints d'IOA et la collection des 100 souches de *S. aureus* correspondantes. Pour chaque couple patient-souche, différents items cliniques incluant le délai d'évolution de l'IOA (délai entre le début des symptômes et le diagnostic microbiologique) sont collectés à l'aide d'un questionnaire standardisé, et mis en regard des données microbiologiques obtenues ex vivo dont la capacité d'invasion de la souche. La présence d'une association significative entre le délai d'évolution et la capacité d'invasion de la souche, observée sur une collection présentant une diversité génétique élevée, apporterait ainsi la démonstration formelle de la pertinence clinique de l'invasion des ostéoblastes par *S. aureus*. Cette démonstration pourrait constituer le socle de connaissance requis pour la mise en place à terme d'une étude multicentrique évaluant le bénéfice de stratégies thérapeutiques adaptées au délai d'évolution de l'IOA à la date de prise en charge.

VI.B. Contribution des PSM à la pathogénie des IOA à CA-MRSA

Nos résultats indiquent que l'invasion des ostéoblastes par les CA-MRSA relève moins d'une stratégie de sanctuarisation que d'une stratégie agressive basée sur l'induction de la mort de la cellule infectée via l'expression des PSM.

Une première question est de savoir si le niveau d'expression des PSM est corrélé à la cytotoxicité de chaque souche de *S. aureus*. En effet, nos conclusions sur le rôle des PSM, bien qu'elles soient appuyés par un faisceau d'arguments solides issus de la littérature, découlent d'observations menées chez le seul fond génétique USA300. Il apparaît donc nécessaire d'étendre le champ de ces conclusions à l'ensemble des CA-MRSA et aux MSSA hypervirulents. Nous travaillons actuellement à définir le niveau d'expression des PSM par RT-PCR quantitative chez les 35 souches de CA-MRSA et HA-MRSA étudiées au cours de nos travaux, dans le but de rechercher une corrélation entre ce niveau d'expression et la cytotoxicité vis-à-vis des ostéoblastes. De plus, le niveau d'expression des PSM sera déterminé chez les 100 souches de l'étude clinico-microbiologique décrite ci-dessus, et une association sera recherchée entre cette expression, la cytotoxicité observée ex vivo et la présentation clinique de l'IOA (délai, sévérité). La confirmation d'une telle association autant chez les MRSA que chez les MSSA permettrait d'établir un rôle des PSM dans la cytotoxicité chez l'ensemble des souches de *S. aureus* les exprimant à haut niveau.

La seconde question a trait à la contribution relative des PSM et de la PVL dans la pathogénie des IOA à CA-MRSA. Le rôle de la PVL dans la destruction tissulaire est indiscutable et s'appuie autant sur des liens épidémiologiques forts entre PVL et gravité que sur un modèle d'ostéomyélite expérimentale (Dohin *et al.*, 2007; Cremieux *et al.*, 2009). Pour déterminer les caractéristiques physiopathologiques propres à chacune de ces toxines, nous nous baserons sur une comparaison de souches isogéniques dans un modèle d'ostéomyélite chez le lapin analogue à celui développé par Crémieux et al. pour démontrer le rôle de la PVL. Les animaux seront infectés par la souche USA300 SF8300 sauvage, ses mutants

isogéniques Δ*pvl*, Δ*psm*, et un double mutant Δ*pvl-psm*. Cette approche, basée sur des paramètres radiologiques et histologiques, permettra de déterminer dans quelle mesure les PSM et la PVL sont responsables de la destruction tissulaire lors des IOA à CA-MRSA.

VI.C. Rôle de l'alpha-toxine dans l'induction de la mort chez les cellules eucaryotes infectées par *S. aureus*

La délétion du gène *hla* codant l'α-toxine chez les souches de CA-MRSA USA300 LAC et SF8300 est associée à une augmentation de cytotoxicité vis-à-vis des ostéoblastes ex vivo. La compréhension des mécanismes associés à ce phénomène inattendu apporterait un gain de connaissance précieux sur la pathogénie intracellulaire de *S. aureus*. Comme décrit précédemment, l'inhibition par l'α-toxine de la β-toxine, elle-même impliquée dans l'échappement phagosomal, pourrait expliquer ce phénomène chez les souches de laboratoire *hlb*+ comme RN4220 (Giese *et al.*, 2011), mais pas chez les souches USA300 exprimant, comme les autres CA-MRSA, une β-toxine non fonctionnelle. Giese et al. ont montré que l'échappement phagosomal était associé à l'expression des PSM de type β, alors que *S. aureus* exprimant les PSM de type α restait confiné dans le phagosome (Giese *et al.*, 2011). Une autre étude a montré que l'induction de la mort chez les polynucléaire neutrophiles ayant phagocyté une souche USA300 survenait sans échappement phagosomal (Kobayashi *et al.*, 2010). L'analyse structurale par microscopie électronique à transmission des neutrophiles infectés indique en effet que la membrane des phagosomes contenant *S. aureus* conserve son intégrité jusqu'à la lyse du leucocyte. En faisant l'hypothèse que les CA-MRSA USA300 induisent la mort de façon similaire et PSM-α-dépendante chez les polynucléaires neutrophiles et chez les ostéoblastes, nous spéculons que les PSM-α exercent leur action indépendamment de l'échappement phagosomal de la bactérie. Nous

proposons cependant que le relargage des PSM α dans le cytoplasme cellulaire est nécessaire à l'induction de mort. Les PSM α sont des protéines de très petite taille (Wang *et al.*, 2007). En conséquence, il est probable que la formation de pores dans la membrane phagosomale, sans nécessairement aboutir à la destruction du phagosome, soit suffisante pour permettre leur passage dans le cytoplasme. En conséquent, une interaction de l'α-toxine avec des facteurs impliqués dans la perméabilisation du phagosome pourrait expliquer en quoi cette toxine est associée à une perte de cytotoxicité. Pour investiguer cette question, nous utiliserons une approche par imagerie confocale et électronique permettant de détecter les variations de perméabilisation de la membrane phagosomale, adaptée des travaux de Giese et al (Giese *et al.*, 2009; Giese *et al.*, 2011). Quatre techniques complémentaires seront employées ; la première consistera à ajouter au milieu de culture un marqueur fluorescent en phase liquide, appelé colorant cargo, lors de la phase d'infection. Le colorant cargo est endocyté conjointement à *S. aureus*. La perméabilisation du phagosome sera alors objectivée par la fuite de ce colorant cargo dans le cytoplasme cellulaire. La seconde technique consistera à employer comme colorant cargo un fluorophore sensible au pH. Le maintien de l'étanchéité du phagosome sera objectivée par un décalage de fluorescence associée à l'acidification du phagosome lors de la fusion lysosomale. Enfin, la troisième technique consistera à faire exprimer par transfection dans le cytoplasme des ostéoblastes une protéine chimère, dite YFP-Fc, comportant un peptide fluorescent couplé à un fragment constant d'IgG. La perméabilisation du phagosome sera objectivée par le recrutement du YFP-Fc sur la paroi bactérienne, en tirant partie de l'affinité de la protéine A pour la fraction Fc des IgG. Enfin, la microscopie électronique à transmission permettra de visualiser l'intégrité ultrastructurale du phagosome. Ces approches combinées permettront de discriminer entre perméabilisation phagosomale d'une part (l'intégrité ultrastructurale du phagosome est conservée malgré une fuite de colorant cargo, un défaut d'acidification et un éventuel recrutement du YFP-Fc si les pores sont de diamètre suffisant), et échappement phagosomal d'autre part (perte d'intégrité du phagosome et présence de *S. aureus* à l'état libre dans le cytoplasme). L'utilisation dans ce modèle de souches isogéniques délétées et recomplémentées pour *hla*, *hld, psm*α et *psm*β permettra de discriminer les rôles respectifs de ces toxines dans la perméabilisation

ou la destruction du phagosome. Sous l'hypothèse de travail, l'expression de *hla* serait associée à un défaut de perméabilisation du phagosome.

La confirmation de ce rôle ambivalent de l'α-toxine dans l'invasion et la cytotoxicité remettrait en question la nature de l'association entre le caractère hémolytique d'une souche donnée et sa capacité à induire la mort cellulaire. Dans le modèle classique, cette association est considérée comme causale : l'hémolyse phénotypique est associée à la production d'α-toxine, et cette production est elle-même responsable de l'induction de mort (Haslinger-Loffler *et al.*, 2005). Nous proposons un modèle indirect : l'hémolyse phénotypique est un marqueur d'activité *agr* élevée, et cette activité est associée à une production élevée de PSM elle-même responsable de l'induction de mort, alors que l'α-toxine joue un rôle d'interférence dans ce phénotype cytotoxique chez les souches hypervirulentes comme les CA-MRSA USA300.

VI.D. Mécanismes moléculaires de la surexpression de toxines chez les *S. aureus* hypervirulents dont les CA-MRSA

La virulence des CA-MRSA est de nature multifactorielle, et combine la présence d'une toxine spécifique, la PVL, et l'expression à haut niveau de toxines codées par le génome cœur de *S. aureus* comme l'α-toxine et les PSM (Otto, 2010). Notre observation du rôle des PSM dans la mort des ostéoblastes infectés par les CA-MRSA est en faveur de cette vision multifactorielle. Certains travaux récents ont montré que la surexpression de toxine chez les CA-MRSA est associée à un niveau d'expression élevé de régulateurs de virulence comme *agr*, *sar*A ou *sae*RS (Li *et al.*, 2010; Montgomery *et al.*, 2010; Otto, 2010; Schlievert *et al.*, 2010). Une question cruciale émerge de ces observations, qui est celle de l'origine de cette surexpression des régulateurs de toxine chez ce groupe de souches pourtant polyphylétique (Tristan *et al.*, 2007a). La recherche des caractéristiques génotypiques associées à

la virulence par « surrégulation » chez *S. aureus* s'impose ainsi comme un enjeu majeur, en partie également parce qu'elle dépasse le strict cadre des CA-MRSA *pvl*+ : premièrement, cette surrégulation est présente chez certains CA-MRSA non porteurs de la PVL comme USA500 (Li *et al.*, 2010) ; deuxièmement, la majorité des infections sévères à *S. aureus* hypervirulents dans certains pays comme la France sont le fait de MSSA, non de CA-MRSA (Gillet *et al.*, 2002; Dohin *et al.*, 2007; Rasigade *et al.*, 2010a) ; et troisièmement, les MSSA hypervirulents *pvl*+ représentent très probablement le réservoir à partir duquel émergent les CA-MRSA (Rasigade *et al.*, 2010b). Nous travaillons actuellement sur une approche de génomique comparative pour identifier des gènes de virulence candidats et/ou des polymorphismes intéressant les systèmes de régulation, associés au phénotype cytotoxique dans notre modèle d'infection intracellulaire *ex vivo*.

Plusieurs hypothèses sous-tendent ce projet. Tout d'abord, il est important de rappeler que les CA-MRSA sont retrouvés au sein de plusieurs fonds génétiques : leur groupe est polyphylétique et il est donc assez peu probable que leur émergence soit liée à une même modification du génome central (chromosome hors éléments génétiques mobiles) qui serait survenue chez différentes lignées de façon répétée et rapprochée dans le temps. D'autre part, un même fond génétique comme le *sequence type* (ST) 8 peut contenir des souches peu virulentes (clone HA-MRSA ST8 Lyon), comme des CA-MRSA exceptionnellement virulents (clone épidémique ST8 USA300). Ce qui différencie les CA-MRSA au sein de ce fond génétique est la présence d'éléments génétiques mobiles, acquis par transfert horizontal, comme le phage phiSLT porteur des gènes codant la PVL (Diep *et al.*, 2006a). Un point commun entre les CA-MRSA issus de différentes lignées pourrait être leur capacité accrue à accepter et intégrer de façon stable certains éléments génétiques mobiles. Nous avons en effet observé que le phage portant la PVL s'intégrait préférentiellement chez les fonds génétiques capables d'intégrer également un autre élément génétique mobile, SCC*mec* (Rasigade *et al.*, 2010b). Nous proposons donc que la cytotoxicité accrue des CA-MRSA est liée à la présence d'un ou plusieurs gènes portés par un élément génétique mobile. Ces gènes peuvent être des toxines, agissant de façon éventuellement synergique avec les PSM, ou jouer un rôle indirect en modulant positivement l'expression de toxines.

Pour tester cette hypothèse, nous nous appuyons sur une méthode innovante de génomique comparative permettant la recherche de cadres de lectures dont la présence ou l'absence est significativement corrélée au niveau de cytotoxicité. Les souches étudiées comprennent l'ensemble des *S. aureus* dont le génome est séquencé, annoté et publié (22 souches dont 4 CA-MRSA), augmentées d'une collection de 200 souches ayant fait l'objet d'un séquençage complet dans le cadre d'une collaboration avec le réseau européen EARSS (Grundmann *et al.*, 2010) (H. Grundmann, RIVM, Pays-Bas) et le Wellcome Trust Sanger Institute (P.J. Howden, Royaume-Uni). L'échantillonnage des souches répond à des objectifs de représentativité et de diversité, et vise à inclure un nombre suffisant de CA-MRSA, de HA-MRSA, et de *S. aureus* sensibles *pvl*+ et *pvl*- dans le plus grand nombre possible de fonds génétiques. Cette collection est testée en modèle *ex vivo* d'infection intracellulaire comme décrit précédemment, pour associer à chaque souche un niveau de cytotoxicité. Les génomes sont annotés et comparés en collaboration avec la plateforme MicroScope (Vallenet *et al.*, 2009), et à l'aide de l'outil bioinformatique MUMmer 3.0 (Kurtz *et al.*, 2004; Diep *et al.*, 2006a) pour identifier les cadres de lecture (ORF) présents chez une proportion de souches de 10 à 90%. Pour chacun de ces ORF, les niveaux de cytotoxicité des souches porteuses et non porteuses seront comparés. Chaque ORF dont la présence ou l'absence est significativement associée à une cytotoxicité accrue sera considéré comme un gène candidat. Des recherches d'homologie et d'associations de gènes permettront ensuite de classer ces candidats par intérêt, en identifiant notemment les ORF associés à un élément génétique mobile.

En parallèle de la recherche d'ORF candidats, nous comparerons les séquences des gènes codant les systèmes de régulation. Bien que le caractère polyphylétique des CA-MRSA rende peu probable le fait qu'une même mutation sur un régulateur donné rende compte de leur virulence, il est cependant envisageable que plusieurs mutations différentes, y compris sur des régulateurs différents, puissent induire une augmentation similaire de virulence. Une association entre chaque polymorphisme identifié et le niveau de cytotoxicité sera recherchée de la même façon que pour les ORF, et désignera le cas échéant des polymorphismes candidats.

Les ORF et/ou polymorphismes désignés in silico devront être caractérisés séparément pour déterminer leur rôle dans la cytotoxicité. Chaque ORF d'intérêt fera l'objet d'une construction génétique d'invalidation (dans un fond génétique hypervirulent et porteur de l'ORF) et de complémentation (dans un fond génétique avirulent et non porteur de l'ORF). Cette étape permettra de confirmer le cas échéant que l'ORF est effectivement associé à la cytotoxicité en modèle *ex vivo*.

Pour valider les polymorphismes d'intérêt impliquant les voies de régulation, chaque polymorphisme candidat dans un fond génétique donné sera introduit par mutagénèse dirigée dans une souche de fond génétique proche mais présentant une cytotoxicité limitée (recherche de gain de fonction), et réparé chez une souche cytotoxique (recherche de perte de fonction).

Au final, l'identification des mécanismes à l'origine de la cytotoxicité des *S. aureus* hypervirulents aurait des retombées tant dans le domaine fondamental que dans le domaine thérapeutique, en aidant à concevoir de nouvelles approches ciblant spécifiquement cette forme de virulence.

REFERENCES

AGERER F., LUX S., MICHEL A., ROHDE M., OHLSEN K. et HAUCK C. R. (2005). Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J Cell Sci*, 2005, 118, Pt 10, p. 2189-200.

AGGARWAL B. B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol*, 2003, 3, 9, p. 745-56.

AHMED S., MEGHJI S., WILLIAMS R. J., HENDERSON B., BROCK J. H. et NAIR S. P. (2001). *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun*, 2001, 69, 5, p. 2872-7.

AL ARFAJ A. S. (2008). A prospective study of the incidence and characteristics of septic arthritis in a teaching hospital in Riyadh, Saudi Arabia. *Clin Rheumatol*, 2008, 27, 11, p. 1403-10.

ALEXANDER E. H., RIVERA F. A., MARRIOTT I., ANGUITA J., BOST K. L. et HUDSON M. C. (2003). *Staphylococcus aureus* - induced tumor necrosis factor - related apoptosis - inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts. *BMC Microbiol*, 2003, 3, p. 5.

ANGEL N. Z., WALSH N., FORWOOD M. R., OSTROWSKI M. C., CASSADY A. I. et HUME D. A. (2000). Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. *J Bone Miner Res*, 2000, 15, 1, p. 103-10.

AREND W. P., PALMER G. et GABAY C. (2008). IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev*, 2008, 223, p. 20-38.

ARNOLD S. R., ELIAS D., BUCKINGHAM S. C., THOMAS E. D., NOVAIS E., ARKADER A. et HOWARD C. (2006). Changing patterns of acute hematogenous osteomyelitis and septic arthritis: emergence of community-associated methicillin-resistant *Staphylococcus aureus*. *J Pediatr Orthop*, 2006, 26, 6, p. 703-8.

ASSUMA R., OATES T., COCHRAN D., AMAR S. et GRAVES D. T. (1998). IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *J Immunol*, 1998, 160, 1, p. 403-9.

AZUMA Y., KAJI K., KATOGI R., TAKESHITA S. et KUDO A. (2000). Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J Biol Chem*, 2000, 275, 7, p. 4858-64.

BABA T., TAKEUCHI F., KURODA M., YUZAWA H., AOKI K., OGUCHI A., NAGAI Y., IWAMA N., ASANO K., NAIMI T., KURODA H., CUI L., YAMAMOTO K. et HIRAMATSU K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, 2002, 359, 9320, p. 1819-27.

BADIOU C., DUMITRESCU O., CROZE M., GILLET Y., DOHIN B., SLAYMAN D. H., ALLAOUCHICHE B., ETIENNE J., VANDENESCH F. et LINA G. (2008). Panton-Valentine leukocidin is expressed at toxic levels in human skin abscesses. *Clin Microbiol Infect*, 2008, 14, 12, p. 1180-3.

BANTEL H., SINHA B., DOMSCHKE W., PETERS G., SCHULZE-OSTHOFF K. et JANICKE R. U. (2001). Alpha-toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J Cell Biol*, 2001, 155, 4, p. 637-48.

BARBIER F., RUPPE E., HERNANDEZ D., LEBEAUX D., FRANCOIS P., FELIX B., DESPREZ A., MAIGA A., WOERTHER P. L., GAILLARD K., JEANROT C., WOLFF M., SCHRENZEL J., ANDREMONT A. et RUIMY R. (2010). Methicillin-resistant coagulase-negative staphylococci in the community: high homology of SCC*mec* IVa between *Staphylococcus epidermidis* and major clones of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis*, 2010, 202, 2, p. 270-81.

BAYER M. G., HEINRICHS J. H. et CHEUNG A. L. (1996). The molecular architecture of the *sar* locus in *Staphylococcus aureus*. *J Bacteriol*, 1996, 178, 15, p. 4563-70.

BAYLES K. W., WESSON C. A., LIOU L. E., FOX L. K., BOHACH G. A. et TRUMBLE W. R. (1998). Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun*, 1998, 66, 1, p. 336-42.

BERGER T. M., HIRSCH E., DJONOV V. et SCHITTNY J. C. (2003). Loss of beta1-integrin-deficient cells during the development of endoderm-derived epithelia. *Anat Embryol (Berl)*, 2003, 207, 4-5, p. 283-8.

BERNARDO K., PAKULAT N., FLEER S., SCHNAITH A., UTERMOHLEN O., KRUT O., MULLER S. et KRONKE M. (2004). Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob Agents Chemother*, 2004, 48, 2, p. 546-55.

BEVERIDGE T. J., MAKIN S. A., KADURUGAMUWA J. L. et LI Z. (1997). Interactions between biofilms and the environment. *FEMS Microbiol Rev*, 1997, 20, 3-4, p. 291-303.

BHAKDI S. et TRANUM-JENSEN J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol Rev*, 1991, 55, 4, p. 733-51.

BINGHAM R. J., RUDINO-PINERA E., MEENAN N. A., SCHWARZ-LINEK U., TURKENBURG J. P., HOOK M., GARMAN E. F. et POTTS J. R. (2008). Crystal structures of fibronectin-binding sites from *Staphylococcus aureus* FnBPA in complex with fibronectin domains. *Proc Natl Acad Sci U S A*, 2008, 105, 34, p. 12254-8.

BLAIR H. C. (1998). How the osteoclast degrades bone. *Bioessays*, 1998, 20, 10, p. 837-46.

BLAIR H. C., TEITELBAUM S. L., GHISELLI R. et GLUCK S. (1989). Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science*, 1989, 245, 4920, p. 855-7.

BLYTH M. J., KINCAID R., CRAIGEN M. A. et BENNET G. C. (2001). The changing epidemiology of acute and subacute haematogenous osteomyelitis in children. *J Bone Joint Surg Br*, 2001, 83, 1, p. 99-102.

BOCCHINI C. E., HULTEN K. G., MASON E. O., JR., GONZALEZ B. E., HAMMERMAN W. A. et KAPLAN S. L. (2006). Panton-Valentine leukocidin genes are associated with enhanced inflammatory response and local disease in acute hematogenous *Staphylococcus aureus* osteomyelitis in children. *Pediatrics*, 2006, 117, 2, p. 433-40.

BOSSARD M. J., TOMASZEK T. A., THOMPSON S. K., AMEGADZIE B. Y., HANNING C. R., JONES C., KURDYLA J. T., MCNULTY D. E., DRAKE F. H., GOWEN M. et LEVY M. A. (1996). Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification. *J Biol Chem*, 1996, 271, 21, p. 12517-24.

BOSSE M. J., GRUBER H. E. et RAMP W. K. (2005). Internalization of bacteria by osteoblasts in a patient with recurrent, long-term osteomyelitis. A case report. *J Bone Joint Surg Am*, 2005, 87, 6, p. 1343-7.

BOST K. L., RAMP W. K., NICHOLSON N. C., BENTO J. L., MARRIOTT I. et HUDSON M. C. (1999). *Staphylococcus aureus* infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production. *J Infect Dis*, 1999, 180, 6, p. 1912-20.

BOULTER E. et VAN OBBERGHEN-SCHILLING E. (2006). Integrin-linked kinase and its partners: a modular platform regulating cell-matrix adhesion dynamics and cytoskeletal organization. *Eur J Cell Biol*, 2006, 85, 3-4, p. 255-63.

BOYCE B. F. et XING L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys*, 2008, 473, 2, p. 139-46.

BOYD A. et CHAKRABARTY A. M. (1994). Role of alginate lyase in cell detachment of Pseudomonas aeruginosa. *Appl Environ Microbiol*, 1994, 60, 7, p. 2355-9.

BOYDEN E. D. et DIETRICH W. F. (2006). Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet*, 2006, 38, 2, p. 240-4.

BOYLE-VAVRA S. et DAUM R. S. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab Invest*, 2007, 87, 1, p. 3-9.

BU R., BORYSENKO C. W., LI Y., CAO L., SABOKBAR A. et BLAIR H. C. (2003). Expression and function of TNF-family proteins and receptors in human osteoblasts. *Bone*, 2003, 33, 5, p. 760-70.

CAMPBELL K. M., VAUGHN A. F., RUSSELL K. L., SMITH B., JIMENEZ D. L., BARROZO C. P., MINARCIK J. R., CRUM N. F. et RYAN M. A. (2004). Risk factors for community-associated methicillin-resistant *Staphylococcus aureus* infections in an outbreak of disease among military trainees in San Diego, California, in 2002. *J Clin Microbiol*, 2004, 42, 9, p. 4050-3.

CANALIS E. (1987). Effects of tumor necrosis factor on bone formation in vitro. *Endocrinology*, 1987, 121, 5, p. 1596-604.

CAREK P. J., DICKERSON L. M. et SACK J. L. (2001). Diagnosis and management of osteomyelitis. *Am Fam Physician*, 2001, 63, 12, p. 2413-20.

CARROLL J. D., CAFFERKEY M. T. et COLEMAN D. C. (1993). Serotype F double- and triple-converting phage insertionally inactivate the *Staphylococcus aureus* beta-toxin determinant by a common molecular mechanism. *FEMS Microbiol Lett*, 1993, 106, 2, p. 147-55.

CENTERS FOR DISEASE CONTROL AND PREVENTION (1999). Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus - Minnesota and North Dakota, 1997-1999. *MMWR Morb Mortal Wkly Rep*, 1999, 48, 32, p. 707-10.

CENTERS FOR DISEASE CONTROL AND PREVENTION (2001). Methicillin-resistant *Staphylococcus aureus* skin or soft tissue infections in a state prison--Mississippi, 2000. *MMWR Morb Mortal Wkly Rep*, 2001, 50, 42, p. 919-22.

CENTERS FOR DISEASE CONTROL AND PREVENTION (2003). Methicillin-resistant *Staphylococcus aureus* infections among competitive sports

participants - Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000-2003. *Conn Med*, 2003, 67, 9, p. 549-51.

CENTRELLA M., MCCARTHY T. L. et CANALIS E. (1988). Tumor necrosis factor-alpha inhibits collagen synthesis and alkaline phosphatase activity independently of its effect on deoxyribonucleic acid synthesis in osteoblast-enriched bone cell cultures. *Endocrinology*, 1988, 123, 3, p. 1442-8.

CHEUNG A. L., BAYER M. G. et HEINRICHS J. H. (1997). *sar* Genetic determinants necessary for transcription of RNAII and RNAIII in the *agr* locus of *Staphylococcus aureus*. *J Bacteriol*, 1997, 179, 12, p. 3963-71.

CHIANG C. Y., KYRITSIS G., GRAVES D. T. et AMAR S. (1999). Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. *Infect Immun*, 1999, 67, 8, p. 4231-6.

CHIEN Y., MANNA A. C. et CHEUNG A. L. (1998). SarA level is a determinant of *agr* activation in *Staphylococcus aureus*. *Mol Microbiol*, 1998, 30, 5, p. 991-1001.

CHIEN Y., MANNA A. C., PROJAN S. J. et CHEUNG A. L. (1999). SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. *J Biol Chem*, 1999, 274, 52, p. 37169-76.

CIAMPOLINI J. et HARDING K. G. (2000). Pathophysiology of chronic bacterial osteomyelitis. Why do antibiotics fail so often? *Postgrad Med J*, 2000, 76, 898, p. 479-83.

CLARKE B. (2008). Normal bone anatomy and physiology. *Clin J Am Soc Nephrol*, 2008, 3 Suppl 3, p. S131-9.

COLIN D. A. et MONTEIL H. (2003). Control of the oxidative burst of human neutrophils by staphylococcal leukotoxins. *Infect Immun*, 2003, 71, 7, p. 3724-9.

COOMBS G. W., NIMMO G. R., PEARSON J. C., CHRISTIANSEN K. J., BELL J. M., COLLIGNON P. J. et MCLAWS M. L. (2009). Prevalence of MRSA strains among *Staphylococcus aureus* isolated from outpatients, 2006. *Commun Dis Intell*, 2009, 33, 1, p. 10-20.

COSTERTON J. W., LEWANDOWSKI Z., CALDWELL D. E., KORBER D. R. et LAPPIN-SCOTT H. M. (1995). Microbial biofilms. *Annu Rev Microbiol*, 1995, 49, p. 711-45.

CREAGH E. M. et O'NEILL L. A. (2006). TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol*, 2006, 27, 8, p. 352-7.

CREMIEUX A. C., DUMITRESCU O., LINA G., VALLEE C., COTE J. F., MUFFAT-JOLY M., LILIN T., ETIENNE J., VANDENESCH F. et SALEH-MGHIR A. (2009). Panton-valentine leukocidin enhances the severity of community-associated methicillin-resistant *Staphylococcus aureus* rabbit osteomyelitis. *PLoS One*, 2009, 4, 9, p. e7204.

DAUWALDER O., LINA G., DURAND G., BES M., MEUGNIER H., JARLIER V., COIGNARD B., VANDENESCH F., ETIENNE J. et LAURENT F. (2008). Epidemiology of invasive methicillin-resistant *Staphylococcus aureus* clones collected in France in 2006 and 2007. *J Clin Microbiol*, 2008, 46, 10, p. 3454-8.

DE BEER D., SRINIVASAN R. et STEWART P. S. (1994). Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol*, 1994, 60, 12, p. 4339-44.

DE BENTZMANN S., TRISTAN A., ETIENNE J., BROUSSE N., VANDENESCH F. et LINA G. (2004). *Staphylococcus aureus* isolates associated with necrotizing pneumonia bind to basement membrane type I and IV collagens and laminin. *J Infect Dis*, 2004, 190, 8, p. 1506-15.

DERETIC V. (2011). Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev*, 2011, 240, 1, p. 92-104.

DIEP B. A., CARLETON H. A., CHANG R. F., SENSABAUGH G. F. et PERDREAU-REMINGTON F. (2006a). Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant Staphylococcus aureus. *J Infect Dis*, 2006a, 193, 11, p. 1495-503.

DIEP B. A., GILL S. R., CHANG R. F., PHAN T. H., CHEN J. H., DAVIDSON M. G., LIN F., LIN J., CARLETON H. A., MONGODIN E. F., SENSABAUGH G. F. et PERDREAU-REMINGTON F. (2006b). Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet*, 2006b, 367, 9512, p. 731-9.

DIEP B. A. et OTTO M. (2008). The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol*, 2008, 16, 8, p. 361-9.

DIEP B. A., STONE G. G., BASUINO L., GRABER C. J., MILLER A., DES ETAGES S. A., JONES A., PALAZZOLO-BALLANCE A. M., PERDREAU-REMINGTON F., SENSABAUGH G. F., DELEO F. R. et CHAMBERS H. F. (2008). The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis*, 2008, 197, 11, p. 1523-30.

DINARELLO C. A. (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol*, 2009, 27, p. 519-50.

DOHIN B., GILLET Y., KOHLER R., LINA G., VANDENESCH F., VANHEMS P., FLORET D. et ETIENNE J. (2007). Pediatric bone and joint infections caused by Panton-Valentine leukocidin-positive *Staphylococcus aureus*. *Pediatr Infect Dis J*, 2007, 26, 11, p. 1042-8.

DRAKE F. H., DODDS R. A., JAMES I. E., CONNOR J. R., DEBOUCK C., RICHARDSON S., LEE-RYKACZEWSKI E., COLEMAN L., RIEMAN D., BARTHLOW R., HASTINGS G. et GOWEN M. (1996). Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J Biol Chem*, 1996, 271, 21, p. 12511-6.

DUBOST J. J., SOUBRIER M., DE CHAMPS C., RISTORI J. M., BUSSIERE J. L. et SAUVEZIE B. (2002). No changes in the distribution of organisms responsible for septic arthritis over a 20 year period. *Ann Rheum Dis*, 2002, 61, 3, p. 267-9.

DUMITRESCU O., BOISSET S., BADIOU C., BES M., BENITO Y., REVERDY M. E., VANDENESCH F., ETIENNE J. et LINA G. (2007). Effect of antibiotics on *Staphylococcus aureus* producing Panton-Valentine leukocidin. *Antimicrob Agents Chemother*, 2007, 51, 4, p. 1515-9.

DZIEWANOWSKA K., PATTI J. M., DEOBALD C. F., BAYLES K. W., TRUMBLE W. R. et BOHACH G. A. (1999). Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun*, 1999, 67, 9, p. 4673-8.

EDWARDS A. M., POTTS J. R., JOSEFSSON E. et MASSEY R. C. (2010). *Staphylococcus aureus* host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. *PLoS Pathog*, 2010, 6, 6, p. e1000964.

ELLINGTON J. K., HARRIS M., HUDSON M. C., VISHIN S., WEBB L. X. et SHERERTZ R. (2006). Intracellular *Staphylococcus aureus* and antibiotic resistance: implications for treatment of staphylococcal osteomyelitis. *J Orthop Res*, 2006, 24, 1, p. 87-93.

ELLINGTON J. K., HARRIS M., WEBB L., SMITH B., SMITH T., TAN K. et HUDSON M. (2003). Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. *J Bone Joint Surg Br*, 2003, 85, 6, p. 918-21.

ELLINGTON J. K., REILLY S. S., RAMP W. K., SMELTZER M. S., KELLAM J. F. et HUDSON M. C. (1999). Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. *Microb Pathog*, 1999, 26, 6, p. 317-23.

ESPERSEN F., FRIMODT-MOLLER N., THAMDRUP ROSDAHL V., SKINHOJ P. et BENTZON M. W. (1991). Changing pattern of bone and joint infections due to Staphylococcus aureus: study of cases of bacteremia in Denmark, 1959-1988. *Rev Infect Dis*, 1991, 13, 3, p. 347-58.

ESPOSITO S. et LEONE S. (2008). Prosthetic joint infections: microbiology, diagnosis, management and prevention. *Int J Antimicrob Agents*, 2008, 32, 4, p. 287-93.

EVANS C. A., JELLIS J., HUGHES S. P., REMICK D. G. et FRIEDLAND J. S. (1998). Tumor necrosis factor-alpha, interleukin-6, and interleukin-8 secretion and the acute-phase response in patients with bacterial and tuberculous osteomyelitis. *J Infect Dis*, 1998, 177, 6, p. 1582-7.

FASSLER R. et MEYER M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev*, 1995, 9, 15, p. 1896-908.

FAUSTIN B., LARTIGUE L., BRUEY J. M., LUCIANO F., SERGIENKO E., BAILLY-MAITRE B., VOLKMANN N., HANEIN D., ROUILLER I. et REED J. C. (2007). Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell*, 2007, 25, 5, p. 713-24.

FENG Y., CHEN C. J., SU L. H., HU S., YU J. et CHIU C. H. (2008). Evolution and pathogenesis of *Staphylococcus aureus*. *FEMS Microbiol Rev*, 2008, 32, 1, p. 23-37.

FOWLER T., WANN E. R., JOH D., JOHANSSON S., FOSTER T. J. et HOOK M. (2000). Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. *Eur J Cell Biol*, 2000, 79, 10, p. 672-9.

FRANCHI L., AMER A., BODY-MALAPEL M., KANNEGANTI T. D., OZOREN N., JAGIRDAR R., INOHARA N., VANDENABEELE P., BERTIN J., COYLE A., GRANT E. P. et NUNEZ G. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol*, 2006, 7, 6, p. 576-82.

FRIDKIN S. K., HAGEMAN J. C., MORRISON M., SANZA L. T., COMO-SABETTI K., JERNIGAN J. A., HARRIMAN K., HARRISON L. H., LYNFIELD R. et FARLEY M. M. (2005). Methicillin-resistant *Staphylococcus aureus* disease in three communities. *N Engl J Med*, 2005, 352, 14, p. 1436-44.

GALLIE W. E. (1951). First recurrence of osteomyelitis eighty years after infection. *J Bone Joint Surg Br*, 1951, 33-B, 1, p. 110-1.

GARCIA-ALVAREZ F., NAVARRO-ZORRAQUINO M., CASTRO A., GRASA J. M., PASTOR C., MONZON M., MARTINEZ A., GARCIA-ALVAREZ I., CASTILLO J. et LOZANO R. (2009). Effect of age on cytokine response in an experimental model of osteomyelitis. *Biogerontology*, 2009, 10, 5, p. 649-58.

GARZONI C. et KELLEY W. L. (2009). *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol*, 2009, 17, 2, p. 59-65.

GENESTIER A. L., MICHALLET M. C., PREVOST G., BELLOT G., CHALABREYSSE L., PEYROL S., THIVOLET F., ETIENNE J., LINA G., VALLETTE F. M., VANDENESCH F. et GENESTIER L. (2005). *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *J Clin Invest*, 2005, 115, 11, p. 3117-27.

GERBER J. S., COFFIN S. E., SMATHERS S. A. et ZAOUTIS T. E. (2009). Trends in the incidence of methicillin-resistant *Staphylococcus aureus* infection in children's hospitals in the United States. *Clin Infect Dis*, 2009, 49, 1, p. 65-71.

GIESE B., DITTMANN S., PAPROTKA K., LEVIN K., WELTROWSKI A., BIEHLER D., LAM T. T., SINHA B. et FRAUNHOLZ M. J. (2009). Staphylococcal alpha-toxin is not sufficient to mediate escape from phagolysosomes in upper-airway epithelial cells. *Infect Immun*, 2009, 77, 9, p. 3611-25.

GIESE B., GLOWINSKI F., PAPROTKA K., DITTMANN S., STEINER T., SINHA B. et FRAUNHOLZ M. J. (2011). Expression of delta-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of beta-toxin. *Cell Microbiol*, 2011, 13, 2, p. 316-29.

GILLESPIE W. J. (1990). Epidemiology in bone and joint infection. *Infect Dis Clin North Am*, 1990, 4, 3, p. 361-76.

GILLET Y., ISSARTEL B., VANHEMS P., FOURNET J. C., LINA G., BES M., VANDENESCH F., PIEMONT Y., BROUSSE N., FLORET D. et ETIENNE J. (2002). Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet*, 2002, 359, 9308, p. 753-9.

GIRAUDO A. T., CALZOLARI A., CATALDI A. A., BOGNI C. et NAGEL R. (1999). The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. *FEMS Microbiol Lett*, 1999, 177, 1, p. 15-22.

GIRAUDO A. T., CHEUNG A. L. et NAGEL R. (1997). The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. *Arch Microbiol*, 1997, 168, 1, p. 53-8.

GOLDENBERG D. L. (1998). Septic arthritis. *Lancet*, 1998, 351, 9097, p. 197-202.

GOMEZ M. I., LEE A., REDDY B., MUIR A., SOONG G., PITT A., CHEUNG A. et PRINCE A. (2004). *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med*, 2004, 10, 8, p. 842-8.

GONZALEZ B. E., MARTINEZ-AGUILAR G., HULTEN K. G., HAMMERMAN W. A., COSS-BU J., AVALOS-MISHAAN A., MASON E. O., JR. et KAPLAN S. L. (2005). Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant Staphylococcus aureus. *Pediatrics*, 2005, 115, 3, p. 642-8.

GONZALEZ B. E., RUEDA A. M., SHELBURNE S. A., 3RD, MUSHER D. M., HAMILL R. J. et HULTEN K. G. (2006a). Community-associated strains of methicillin-resistant *Staphylococccus aureus* as the cause of healthcare-associated infection. *Infect Control Hosp Epidemiol*, 2006a, 27, 10, p. 1051-6.

GONZALEZ B. E., TERUYA J., MAHONEY D. H., JR., HULTEN K. G., EDWARDS R., LAMBERTH L. B., HAMMERMAN W. A., MASON E. O., JR. et KAPLAN S. L. (2006b). Venous thrombosis associated with staphylococcal osteomyelitis in children. *Pediatrics*, 2006b, 117, 5, p. 1673-9.

GRAMMATICO L., BARON S., RUSCH E., LEPAGE B., SURER N., DESENCLOS J. C. et BESNIER J. M. (2008). Epidemiology of vertebral osteomyelitis (VO) in France: analysis of hospital-discharge data 2002-2003. *Epidemiol Infect*, 2008, 136, 5, p. 653-60.

GREER R. B. et ROSENBERG A. E. (1993). Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 6-1993. A 69-year-old woman with a sclerotic lesion of the femur and pulmonary nodules. *N Engl J Med*, 1993, 328, 6, p. 422-8.

GREIG J. M. et WOOD M. J. (2003). *Staphylococcus lugdunensis* vertebral osteomyelitis. *Clin Microbiol Infect*, 2003, 9, 11, p. 1139-41.

GROJEC P. L. et JELJASZEWICZ J. (1981). Effect of staphylococcal leukocidin on mouse leukocyte system. *Zentralbl Bakteriol Mikrobiol Hyg [A]*, 1981, 250, 4, p. 446-55.

GRUNDMANN H., AANENSEN D. M., VAN DEN WIJNGAARD C. C., SPRATT B. G., HARMSEN D. et FRIEDRICH A. W. (2010). Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med*, 2010, 7, 1, p. e1000215.

GWYNNE-JONES D. P. et STOTT N. S. (1999). Community-acquired methicillin-resistant *Staphylococcus aureus*: a cause of musculoskeletal sepsis in children. *J Pediatr Orthop*, 1999, 19, 3, p. 413-6.

HANKIN D., BOWLING F. L., METCALFE S. A., WHITEHOUSE R. A. et BOULTON A. J. (2011). Critically evaluating the role of diagnostic imaging in osteomyelitis. *Foot Ankle Spec*, 2011, 4, 2, p. 100-5.

HARRAGHY N., KORMANEC J., WOLZ C., HOMEROVA D., GOERKE C., OHLSEN K., QAZI S., HILL P. et HERRMANN M. (2005). *sae* is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology*, 2005, 151, Pt 6, p. 1789-800.

HASLINGER B., STRANGFELD K., PETERS G., SCHULZE-OSTHOFF K. et SINHA B. (2003). *Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral

blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. *Cell Microbiol*, 2003, 5, 10, p. 729-41.

HASLINGER-LOFFLER B., KAHL B. C., GRUNDMEIER M., STRANGFELD K., WAGNER B., FISCHER U., CHEUNG A. L., PETERS G., SCHULZE-OSTHOFF K. et SINHA B. (2005). Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. *Cell Microbiol*, 2005, 7, 8, p. 1087-97.

HASLINGER-LOFFLER B., WAGNER B., BRUCK M., STRANGFELD K., GRUNDMEIER M., FISCHER U., VOLKER W., PETERS G., SCHULZE-OSTHOFF K. et SINHA B. (2006). *Staphylococcus aureus* induces caspase-independent cell death in human peritoneal mesothelial cells. *Kidney Int*, 2006, 70, 6, p. 1089-98.

HATTERSLEY G., DOREY E., HORTON M. A. et CHAMBERS T. J. (1988). Human macrophage colony-stimulating factor inhibits bone resorption by osteoclasts disaggregated from rat bone. *J Cell Physiol*, 1988, 137, 1, p. 199-203.

HAUCK C. R. et OHLSEN K. (2006). Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. *Curr Opin Microbiol*, 2006, 9, 1, p. 5-11.

HAYMAN A. R., JONES S. J., BOYDE A., FOSTER D., COLLEDGE W. H., CARLTON M. B., EVANS M. J. et COX T. M. (1996). Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development*, 1996, 122, 10, p. 3151-62.

HENDERSON B. et NAIR S. P. (2003). Hard labour: bacterial infection of the skeleton. *Trends Microbiol*, 2003, 11, 12, p. 570-7.

HENRIKSEN K., NEUTZSKY-WULFF A. V., BONEWALD L. F. et KARSDAL M. A. (2009). Local communication on and within bone controls bone remodeling. *Bone*, 2009, 44, 6, p. 1026-33.

HERBERT S., BARRY P. et NOVICK R. P. (2001). Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect Immun*, 2001, 69, 5, p. 2996-3003.

HERBERT S., ZIEBANDT A. K., OHLSEN K., SCHAFER T., HECKER M., ALBRECHT D., NOVICK R. et GOTZ F. (2010). Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun*, 2010, 78, 6, p. 2877-89.

HEROLD B. C., IMMERGLUCK L. C., MARANAN M. C., LAUDERDALE D. S., GASKIN R. E., BOYLE-VAVRA S., LEITCH C. D. et DAUM R. S. (1998). Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *Jama*, 1998, 279, 8, p. 593-8.

HIKITA A., YANA I., WAKEYAMA H., NAKAMURA M., KADONO Y., OSHIMA Y., NAKAMURA K., SEIKI M. et TANAKA S. (2006). Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. *J Biol Chem*, 2006, 281, 48, p. 36846-55.

HOFFMANN C., OHLSEN K. et HAUCK C. R. (2011). Integrin-mediated uptake of fibronectin-binding bacteria. *Eur J Cell Biol*, 2011, 90, 11, p. 891-6.

HOSHINO K., TAKEUCHI O., KAWAI T., SANJO H., OGAWA T., TAKEDA Y., TAKEDA K. et AKIRA S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*, 1999, 162, 7, p. 3749-52.

HSU L. Y., KOH T. H., TAN T. Y., ITO T., MA X. X., LIN R. T. et TAN B. H. (2006a). Emergence of community-associated methicillin-resistant *Staphylococcus aureus* in Singapore: a further six cases. *Singapore Med J*, 2006a, 47, 1, p. 20-6.

HSU L. Y., KOH Y. L., CHLEBICKA N. L., TAN T. Y., KRISHNAN P., LIN R. T., TEE N., BARKHAM T. et KOH T. H. (2006b). Establishment of ST30 as the predominant clonal type among community-associated methicillin-resistant

Staphylococcus aureus isolates in Singapore. *J Clin Microbiol*, 2006b, 44, 3, p. 1090-3.

HSU L. Y., TRISTAN A., KOH T. H., BES M., ETIENNE J., KURUP A., TAN T. T. et TAN B. H. (2005). Community associated methicillin-resistant *Staphylococcus aureus*, Singapore. *Emerg Infect Dis*, 2005, 11, 2, p. 341-2.

HUANG H., FLYNN N. M., KING J. H., MONCHAUD C., MORITA M. et COHEN S. H. (2006). Comparisons of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) and hospital-associated MSRA infections in Sacramento, California. *J Clin Microbiol*, 2006, 44, 7, p. 2423-7.

HUDSON M. C., RAMP W. K., NICHOLSON N. C., WILLIAMS A. S. et NOUSIAINEN M. T. (1995). Internalization of *Staphylococcus aureus* by cultured osteoblasts. *Microb Pathog*, 1995, 19, 6, p. 409-19.

HUITEMA L. F. et VAANDRAGER A. B. (2007). What triggers cell-mediated mineralization? *Front Biosci*, 2007, 12, p. 2631-45.

HUSEBY M., SHI K., BROWN C. K., DIGRE J., MENGISTU F., SEO K. S., BOHACH G. A., SCHLIEVERT P. M., OHLENDORF D. H. et EARHART C. A. (2007). Structure and biological activities of beta toxin from *Staphylococcus aureus*. *J Bacteriol*, 2007, 189, 23, p. 8719-26.

HUSSAIN M., HAGGAR A., PETERS G., CHHATWAL G. S., HERRMANN M., FLOCK J. I. et SINHA B. (2008). More than one tandem repeat domain of the extracellular adherence protein of *Staphylococcus aureus* is required for aggregation, adherence, and host cell invasion but not for leukocyte activation. *Infect Immun*, 2008, 76, 12, p. 5615-23.

HYBISKE K. et STEPHENS R. S. (2008). Exit strategies of intracellular pathogens. *Nat Rev Microbiol*, 2008, 6, 2, p. 99-110.

ISHIMI Y., MIYAURA C., JIN C. H., AKATSU T., ABE E., NAKAMURA Y., YAMAGUCHI A., YOSHIKI S., MATSUDA T., HIRANO T. et ET AL. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol*, 1990, 145, 10, p. 3297-303.

JANZON L., LOFDAHL S. et ARVIDSON S. (1989). Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol Gen Genet*, 1989, 219, 3, p. 480-5.

JARRAUD S., MOUGEL C., THIOULOUSE J., LINA G., MEUGNIER H., FOREY F., NESME X., ETIENNE J. et VANDENESCH F. (2002). Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect Immun*, 2002, 70, 2, p. 631-41.

JARRY T. M. et CHEUNG A. L. (2006). *Staphylococcus aureus* escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart. *Infect Immun*, 2006, 74, 5, p. 2568-77.

JARRY T. M., MEMMI G. et CHEUNG A. L. (2008). The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in CFT-1 cells. *Cell Microbiol*, 2008, 10, 9, p. 1801-14.

JENNIN F., BOUSSON V., PARLIER C., JOMAAH N., KHANINE V. et LAREDO J. D. (2010). Bony sequestrum: a radiologic review. *Skeletal Radiol*, 2010, 40, 8, p. 963-75.

JEVON M., GUO C., MA B., MORDAN N., NAIR S. P., HARRIS M., HENDERSON B., BENTLEY G. et MEGHJI S. (1999). Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. *Infect Immun*, 1999, 67, 5, p. 2677-81.

JEVONS M. P., COE A. W. et PARKER M. T. (1963). Methicillin resistance in staphylococci. *Lancet*, 1963, 1, 7287, p. 904-7.

JOUBERT O., VOEGELIN J., GUILLET V., TRANIER S., WERNER S., COLIN D. A., SERRA M. D., KELLER D., MONTEIL H., MOUREY L. et PREVOST G. (2007). Distinction between Pore Assembly by Staphylococcal alpha-Toxin versus Leukotoxins. *J Biomed Biotechnol*, 2007, 2007, p. 25935.

KAHLENBERG J. M., LUNDBERG K. C., KERTESY S. B., QU Y. et DUBYAK G. R. (2005). Potentiation of caspase-1 activation by the P2X7 receptor is

dependent on TLR signals and requires NF-kappaB-driven protein synthesis. *J Immunol*, 2005, 175, 11, p. 7611-22.

KAITO C., OMAE Y., MATSUMOTO Y., NAGATA M., YAMAGUCHI H., AOTO T., ITO T., HIRAMATSU K. et SEKIMIZU K. (2008). A novel gene, fudoh, in the SCC*mec* region suppresses the colony spreading ability and virulence of *Staphylococcus aureus*. *PLoS ONE*, 2008, 3, 12, p. e3921.

KANAZAWA K., AZUMA Y., NAKANO H. et KUDO A. (2003). TRAF5 functions in both RANKL- and TNFalpha-induced osteoclastogenesis. *J Bone Miner Res*, 2003, 18, 3, p. 443-50.

KANEKO J. et KAMIO Y. (2004). Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Biosci Biotechnol Biochem*, 2004, 68, 5, p. 981-1003.

KARWOWSKA A., DAVIES H. D. et JADAVJI T. (1998). Epidemiology and outcome of osteomyelitis in the era of sequential intravenous-oral therapy. *Pediatr Infect Dis J*, 1998, 17, 11, p. 1021-6.

KAZAKOVA S. V., HAGEMAN J. C., MATAVA M., SRINIVASAN A., PHELAN L., GARFINKEL B., BOO T., MCALLISTER S., ANDERSON J., JENSEN B., DODSON D., LONSWAY D., MCDOUGAL L. K., ARDUINO M., FRASER V. J., KILLGORE G., TENOVER F. C., CODY S. et JERNIGAN D. B. (2005). A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *N Engl J Med*, 2005, 352, 5, p. 468-75.

KECHRID A., PEREZ-VAZQUEZ M., SMAOUI H., HARIGA D., RODRIGUEZ-BANOS M., VINDEL A., BAQUERO F., CANTON R. et DEL CAMPO R. (2010). Molecular analysis of community-acquired methicillin-susceptible and resistant *Staphylococcus aureus* isolates recovered from bacteraemic and osteomyelitis infections in children from Tunisia. *Clin Microbiol Infect*, 2010, p.

KIM H. H., LEE D. E., SHIN J. N., LEE Y. S., JEON Y. M., CHUNG C. H., NI J., KWON B. S. et LEE Z. H. (1999). Receptor activator of NF-kappaB recruits

multiple TRAF family adaptors and activates c-Jun N-terminal kinase. *FEBS Lett*, 1999, 443, 3, p. 297-302.

KINTARAK S., WHAWELL S. A., SPEIGHT P. M., PACKER S. et NAIR S. P. (2004). Internalization of *Staphylococcus aureus* by human keratinocytes. *Infect Immun*, 2004, 72, 10, p. 5668-75.

KLEIN M., KRONKE M. et KRUT O. (2006). Expression of lysostaphin in HeLa cells protects from host cell killing by intracellular *Staphylococcus aureus*. *Med Microbiol Immunol*, 2006, 195, 3, p. 159-63.

KLEVENS R. M., MORRISON M. A., NADLE J., PETIT S., GERSHMAN K., RAY S., HARRISON L. H., LYNFIELD R., DUMYATI G., TOWNES J. M., CRAIG A. S., ZELL E. R., FOSHEIM G. E., MCDOUGAL L. K., CAREY R. B. et FRIDKIN S. K. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Jama*, 2007, 298, 15, p. 1763-71.

KLOSTERHALFEN B., PETERS K. M., TONS C., HAUPTMANN S., KLEIN C. L. et KIRKPATRICK C. J. (1996). Local and systemic inflammatory mediator release in patients with acute and chronic posttraumatic osteomyelitis. *J Trauma*, 1996, 40, 3, p. 372-8.

KOBAYASHI S. D., BRAUGHTON K. R., PALAZZOLO-BALLANCE A. M., KENNEDY A. D., SAMPAIO E., KRISTOSTURYAN E., WHITNEY A. R., STURDEVANT D. E., DORWARD D. W., HOLLAND S. M., KREISWIRTH B. N., MUSSER J. M. et DELEO F. R. (2010). Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun*, 2010, 2, 6, p. 560-75.

KONIG A., MUHLBAUER R. C. et FLEISCH H. (1988). Tumor necrosis factor alpha and interleukin-1 stimulate bone resorption in vivo as measured by urinary [3H]tetracycline excretion from prelabeled mice. *J Bone Miner Res*, 1988, 3, 6, p. 621-7.

KONIG B., KOLLER M., PREVOST G., PIEMONT Y., ALOUF J. E., SCHREINER A. et KONIG W. (1994). Activation of human effector cells by different bacterial

toxins (leukocidin, alveolysin, and erythrogenic toxin A): generation of interleukin-8. *Infect Immun*, 1994, 62, 11, p. 4831-7.

KONIG B., PREVOST G., PIEMONT Y. et KONIG W. (1995). Effects of *Staphylococcus aureus* leukocidins on inflammatory mediator release from human granulocytes. *J Infect Dis*, 1995, 171, 3, p. 607-13.

KORNAK U., KASPER D., BOSL M. R., KAISER E., SCHWEIZER M., SCHULZ A., FRIEDRICH W., DELLING G. et JENTSCH T. J. (2001). Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell*, 2001, 104, 2, p. 205-15.

KOROVESSIS P., FORTIS A. P., SPASTRIS P. et DROUTSAS P. (1991). Acute osteomyelitis of the patella 50 years after a knee fusion for septic arthritis. A case report. *Clin Orthop Relat Res*, 1991, 272, p. 205-7.

KOTAKE S., SATO K., KIM K. J., TAKAHASHI N., UDAGAWA N., NAKAMURA I., YAMAGUCHI A., KISHIMOTO T., SUDA T. et KASHIWAZAKI S. (1996). Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res*, 1996, 11, 1, p. 88-95.

KRETSCHMER D., GLESKE A. K., RAUTENBERG M., WANG R., KOBERLE M., BOHN E., SCHONEBERG T., RABIET M. J., BOULAY F., KLEBANOFF S. J., VAN KESSEL K. A., VAN STRIJP J. A., OTTO M. et PESCHEL A. (2010). Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. *Cell Host Microbe*, 2010, 7, 6, p. 463-73.

KUBICA M., GUZIK K., KOZIEL J., ZAREBSKI M., RICHTER W., GAJKOWSKA B., GOLDA A., MACIAG-GUDOWSKA A., BRIX K., SHAW L., FOSTER T. et POTEMPA J. (2008). A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One*, 2008, 3, 1, p. e1409.

KURTZ S., PHILLIPPY A., DELCHER A. L., SMOOT M., SHUMWAY M., ANTONESCU C. et SALZBERG S. L. (2004). Versatile and open software for comparing large genomes. *Genome Biol*, 2004, 5, 2, p. R12.

KWAN TAT S., PADRINES M., THEOLEYRE S., HEYMANN D. et FORTUN Y. (2004). IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev*, 2004, 15, 1, p. 49-60.

LABANDEIRA-REY M., COUZON F., BOISSET S., BROWN E. L., BES M., BENITO Y., BARBU E. M., VAZQUEZ V., HOOK M., ETIENNE J., VANDENESCH F. et BOWDEN M. G. (2007). *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science*, 2007, 315, 5815, p. 1130-3.

LACEY D. C., SIMMONS P. J., GRAVES S. E. et HAMILTON J. A. (2009). Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. *Osteoarthritis Cartilage*, 2009, 17, 6, p. 735-42.

LANG S., LIVESLEY M. A., LAMBERT P. A., ELLIOTT J. et ELLIOTT T. S. (1999). The genomic diversity of coagulase-negative staphylococci associated with nosocomial infections. *J Hosp Infect*, 1999, 43, 3, p. 187-93.

LAU Y. S., WANG W., SABOKBAR A., SIMPSON H., NAIR S., HENDERSON B., BERENDT A. et ATHANASOU N. A. (2006). *Staphylococcus aureus* capsular material promotes osteoclast formation. *Injury*, 2006, 37 Suppl 2, p. S41-8.

LAZZARINI L., MADER J. T. et CALHOUN J. H. (2004). Osteomyelitis in long bones. *J Bone Joint Surg Am*, 2004, 86-A, 10, p. 2305-18.

LEE K. et GOODMAN S. B. (2008). Current state and future of joint replacements in the hip and knee. *Expert Rev Med Devices*, 2008, 5, 3, p. 383-93.

LEE S. M., ENDER M., ADHIKARI R., SMITH J. M., BERGER-BACHI B. et COOK G. M. (2007). Fitness cost of staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus aureus* by way of continuous culture. *Antimicrob Agents Chemother*, 2007, 51, 4, p. 1497-9.

LEVINE M. et SIEGEL L. B. (2003). A swollen joint: why all the fuss? *Am J Ther*, 2003, 10, 3, p. 219-24.

LEW D. P. et WALDVOGEL F. A. (1997). Osteomyelitis. *N Engl J Med*, 1997, 336, 14, p. 999-1007.

LEW D. P. et WALDVOGEL F. A. (2004). Osteomyelitis. *Lancet*, 2004, 364, 9431, p. 369-79.

LI M., CHEUNG G. Y., HU J., WANG D., JOO H. S., DELEO F. R. et OTTO M. (2010). Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. *J Infect Dis*, 2010, 202, 12, p. 1866-76.

LI Y. P. et STASHENKO P. (1992). Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter. *J Immunol*, 1992, 148, 3, p. 788-94.

LIANG X. et JI Y. (2006). Alpha-toxin interferes with integrin-mediated adhesion and internalization of *Staphylococcus aureus* by epithelial cells. *Cell Microbiol*, 2006, 8, 10, p. 1656-68.

LINA G., PIEMONT Y., GODAIL-GAMOT F., BES M., PETER M. O., GAUDUCHON V., VANDENESCH F. et ETIENNE J. (1999). Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis*, 1999, 29, 5, p. 1128-32.

LJUSBERG J., WANG Y., LANG P., NORGARD M., DODDS R., HULTENBY K., EK-RYLANDER B. et ANDERSSON G. (2005). Proteolytic excision of a repressive loop domain in tartrate-resistant acid phosphatase by cathepsin K in osteoclasts. *J Biol Chem*, 2005, 280, 31, p. 28370-81.

LOMAGA M. A., YEH W. C., SAROSI I., DUNCAN G. S., FURLONGER C., HO A., MORONY S., CAPPARELLI C., VAN G., KAUFMAN S., VAN DER HEIDEN A., ITIE A., WAKEHAM A., KHOO W., SASAKI T., CAO Z., PENNINGER J. M., PAIGE C. J., LACEY D. L., DUNSTAN C. R., BOYLE W. J., GOEDDEL D. V. et MAK T. W. (1999). TRAF6 deficiency results in osteopetrosis and

defective interleukin-1, CD40, and LPS signaling. *Genes Dev*, 1999, 13, 8, p. 1015-24.

LYNCH C. C., HIKOSAKA A., ACUFF H. B., MARTIN M. D., KAWAI N., SINGH R. K., VARGO-GOGOLA T. C., BEGTRUP J. L., PETERSON T. E., FINGLETON B., SHIRAI T., MATRISIAN L. M. et FUTAKUCHI M. (2005). MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Cancer Cell*, 2005, 7, 5, p. 485-96.

MA X. X., ITO T., CHONGTRAKOOL P. et HIRAMATSU K. (2006). Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant Staphylococcus aureus strains isolated in Japanese hospitals from 1979 to 1985. *J Clin Microbiol*, 2006, 44, 12, p. 4515-27.

MACKIE E. J. (2003). Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol*, 2003, 35, 9, p. 1301-5.

MAHALINGAM D., SZEGEZDI E., KEANE M., DE JONG S. et SAMALI A. (2009). TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev*, 2009, 35, 3, p. 280-8.

MAIN C. L., JAYARATNE P., HALEY A., RUTHERFORD C., SMAILL F. et FISMAN D. N. (2005). Outbreaks of infection caused by community-acquired methicillin-resistant *Staphylococcus aureus* in a Canadian correctional facility. *Can J Infect Dis Med Microbiol*, 2005, 16, 6, p. 343-8.

MARIATHASAN S., WEISS D. S., NEWTON K., MCBRIDE J., O'ROURKE K., ROOSE-GIRMA M., LEE W. P., WEINRAUCH Y., MONACK D. M. et DIXIT V. M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*, 2006, 440, 7081, p. 228-32.

MARRIOTT I., HUGHES F. M., JR. et BOST K. L. (2002). Bacterial infection of osteoblasts induces interleukin-1beta and interleukin-18 transcription but not protein synthesis. *J Interferon Cytokine Res*, 2002, 22, 10, p. 1049-55.

MARRIOTT I., RATI D. M., MCCALL S. H. et TRANGUCH S. L. (2005). Induction of Nod1 and Nod2 intracellular pattern recognition receptors in murine

osteoblasts following bacterial challenge. *Infect Immun*, 2005, 73, 5, p. 2967-73.

MARTINEZ-AGUILAR G., AVALOS-MISHAAN A., HULTEN K., HAMMERMAN W., MASON E. O., JR. et KAPLAN S. L. (2004). Community-acquired, methicillin-resistant and methicillin-susceptible Staphylococcus aureus musculoskeletal infections in children. *Pediatr Infect Dis J*, 2004, 23, 8, p. 701-6.

MASSEY R. C., KANTZANOU M. N., FOWLER T., DAY N. P., SCHOFIELD K., WANN E. R., BERENDT A. R., HOOK M. et PEACOCK S. J. (2001). Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol*, 2001, 3, 12, p. 839-51.

MATSUO K. et IRIE N. (2008). Osteoclast-osteoblast communication. *Arch Biochem Biophys*, 2008, 473, 2, p. 201-9.

MCCALL S. H., SAHRAEI M., YOUNG A. B., WORLEY C. S., DUNCAN J. A., TING J. P. et MARRIOTT I. (2008). Osteoblasts express NLRP3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. *J Bone Miner Res*, 2008, 23, 1, p. 30-40.

MCDOUGAL L. K., STEWARD C. D., KILLGORE G. E., CHAITRAM J. M., MCALLISTER S. K. et TENOVER F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol*, 2003, 41, 11, p. 5113-20.

MEGHJI S., CREAN S. J., HILL P. A., SHEIKH M., NAIR S. P., HERON K., HENDERSON B., MAWER E. B. et HARRIS M. (1998). Surface-associated protein from *Staphylococcus aureus* stimulates osteoclastogenesis: possible role in *S. aureus*-induced bone pathology. *Br J Rheumatol*, 1998, 37, 10, p. 1095-101.

MENESTRINA G. (1986). Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage-dependent inhibition by divalent and trivalent cations. *J Membr Biol*, 1986, 90, 2, p. 177-90.

MIAO E. A., ALPUCHE-ARANDA C. M., DORS M., CLARK A. E., BADER M. W., MILLER S. I. et ADEREM A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol*, 2006, 7, 6, p. 569-75.

MILLER L. G., PERDREAU-REMINGTON F., RIEG G., MEHDI S., PERLROTH J., BAYER A. S., TANG A. W., PHUNG T. O. et SPELLBERG B. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J Med*, 2005, 352, 14, p. 1445-53.

MITCHELL G., LAMONTAGNE C. A., BROUILLETTE E., GRONDIN G., TALBOT B. G., GRANDBOIS M. et MALOUIN F. (2008). *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. *Mol Microbiol*, 2008, 70, 6, p. 1540-55.

MONTGOMERY C. P., BOYLE-VAVRA S., ADEM P. V., LEE J. C., HUSAIN A. N., CLASEN J. et DAUM R. S. (2008). Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. *J Infect Dis*, 2008, 198, 4, p. 561-70.

MONTGOMERY C. P., BOYLE-VAVRA S. et DAUM R. S. (2010). Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. *PLoS One*, 2010, 5, 12, p. e15177.

MONZON M., OTEIZA C., LEIVA J., LAMATA M. et AMORENA B. (2002). Biofilm testing of Staphylococcus epidermidis clinical isolates: low performance of vancomycin in relation to other antibiotics. *Diagn Microbiol Infect Dis*, 2002, 44, 4, p. 319-24.

MORAN G. J., KRISHNADASAN A., GORWITZ R. J., FOSHEIM G. E., MCDOUGAL L. K., CAREY R. B. et TALAN D. A. (2006). Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med*, 2006, 355, 7, p. 666-74.

MORRISSY R. T. et HAYNES D. W. (1989). Acute hematogenous osteomyelitis: a model with trauma as an etiology. *J Pediatr Orthop*, 1989, 9, 4, p. 447-56.

MUNCKHOF W. J., SCHOONEVELDT J., COOMBS G. W., HOARE J. et NIMMO G. R. (2003). Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infection in Queensland, Australia. *Int J Infect Dis*, 2003, 7, 4, p. 259-64.

MUNDY G. R. (1991). Inflammatory mediators and the destruction of bone. *J Periodontal Res*, 1991, 26, 3 Pt 2, p. 213-7.

MURDOCH D. R., EVERTS R. J., CHAMBERS S. T. et COWAN I. A. (1996). Vertebral osteomyelitis due to *Staphylococcus lugdunensis*. *J Clin Microbiol*, 1996, 34, 4, p. 993-4.

NADE S. (2003). Septic arthritis. *Best Pract Res Clin Rheumatol*, 2003, 17, 2, p. 183-200.

NAIMI T. S., LEDELL K. H., COMO-SABETTI K., BORCHARDT S. M., BOXRUD D. J., ETIENNE J., JOHNSON S. K., VANDENESCH F., FRIDKIN S., O'BOYLE C., DANILA R. N. et LYNFIELD R. (2003). Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *Jama*, 2003, 290, 22, p. 2976-84.

NAIR S., SONG Y., MEGHJI S., REDDI K., HARRIS M., ROSS A., POOLE S., WILSON M. et HENDERSON B. (1995). Surface-associated proteins from *Staphylococcus aureus* demonstrate potent bone resorbing activity. *J Bone Miner Res*, 1995, 10, 5, p. 726-34.

NANES M. S. (2003). Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology. *Gene*, 2003, 321, p. 1-15.

NANES M. S., MCKOY W. M. et MARX S. J. (1989). Inhibitory effects of tumor necrosis factor-alpha and interferon-gamma on deoxyribonucleic acid and collagen synthesis by rat osteosarcoma cells (ROS 17/2.8). *Endocrinology*, 1989, 124, 1, p. 339-45.

NANES M. S., RUBIN J., TITUS L., HENDY G. N. et CATHERWOOD B. (1991). Tumor necrosis factor-alpha inhibits 1,25-dihydroxyvitamin D3-stimulated bone Gla protein synthesis in rat osteosarcoma cells (ROS 17/2.8) by a pretranslational mechanism. *Endocrinology*, 1991, 128, 5, p. 2577-82.

NATIONAL NOSOCOMIAL INFECTIONS SURVEILLANCE (NNIS) (2004). System Report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control*, 2004, 32, 8, p. 470-85.

NESBITT S. A. et HORTON M. A. (1997). Trafficking of matrix collagens through bone-resorbing osteoclasts. *Science*, 1997, 276, 5310, p. 266-9.

NIMMO G. R., SCHOONEVELDT J., O'KANE G., MCCALL B. et VICKERY A. (2000). Community acquisition of gentamicin-sensitive methicillin-resistant *Staphylococcus aureus* in southeast Queensland, Australia. *J Clin Microbiol*, 2000, 38, 11, p. 3926-31.

NOVICK R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol*, 2003, 48, 6, p. 1429-49.

NYGAARD T. K., PALLISTER K. B., DUMONT A. L., DEWALD M., WATKINS R. L., PALLISTER E. Q., MALONE C., GRIFFITH S., HORSWILL A. R., TORRES V. J. et VOYICH J. M. (2012). Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One*, 2012, 7, 5, p. e36532.

OLIVIER A. C., LEMAIRE S., VAN BAMBEKE F., TULKENS P. M. et OLDFIELD E. (2009). Role of *rsb*U and staphyloxanthin in phagocytosis and intracellular growth of *Staphylococcus aureus* in human macrophages and endothelial cells. *J Infect Dis*, 2009, 200, 9, p. 1367-70.

OSIRI M., RUXRUNGTHAM K., NOOKHAI S., OHMOTO Y. et DEESOMCHOK U. (1998). IL-1beta, IL-6 and TNF-alpha in synovial fluid of patients with non-gonococcal septic arthritis. *Asian Pac J Allergy Immunol*, 1998, 16, 4, p. 155-60.

OTTO M. (2010). Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol*, 2010, 64, p. 143-62.

PARK S. H., PARK C., YOO J. H., CHOI S. M., CHOI J. H., SHIN H. H., LEE D. G., LEE S., KIM J., CHOI S. E., KWON Y. M. et SHIN W. S. (2009). Emergence of community-associated methicillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated bloodstream infections in Korea. *Infect Control Hosp Epidemiol*, 2009, 30, 2, p. 146-55.

PARVIZI J., PAWASARAT I. M., AZZAM K. A., JOSHI A., HANSEN E. N. et BOZIC K. J. (2010). Periprosthetic joint infection: the economic impact of methicillin-resistant infections. *J Arthroplasty*, 2010, 25, 6 Suppl, p. 103-7.

PENG H. L., NOVICK R. P., KREISWIRTH B., KORNBLUM J. et SCHLIEVERT P. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J Bacteriol*, 1988, 170, 9, p. 4365-72.

PFEILSCHIFTER J., CHENU C., BIRD A., MUNDY G. R. et ROODMAN G. D. (1989). Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells in vitro. *J Bone Miner Res*, 1989, 4, 1, p. 113-8.

PIVIROTTO L. A., CISSEL D. S. et KEETING P. E. (1995). Sex hormones mediate interleukin-1 beta production by human osteoblastic HOBIT cells. *Mol Cell Endocrinol*, 1995, 111, 1, p. 67-74.

QAZI S. N., COUNIL E., MORRISSEY J., REES C. E., COCKAYNE A., WINZER K., CHAN W. C., WILLIAMS P. et HILL P. J. (2001). *agr* expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. *Infect Immun*, 2001, 69, 11, p. 7074-82.

RAMDANI-BOUGUESSA N., BES M., MEUGNIER H., FOREY F., REVERDY M. E., LINA G., VANDENESCH F., TAZIR M. et ETIENNE J. (2006). Detection of methicillin-resistant *Staphylococcus aureus* strains resistant to multiple antibiotics and carrying the Panton-Valentine leukocidin genes in an Algiers hospital. *Antimicrob Agents Chemother*, 2006, 50, 3, p. 1083-5.

RASIGADE J. P., LAURENT F., HUBERT P., VANDENESCH F. et ETIENNE J. (2010a). Lethal necrotizing pneumonia caused by an ST398 *Staphylococcus aureus* strain. *Emerg Infect Dis*, 2010a, 16, 8, p. 1330.

RASIGADE J. P., LAURENT F., LINA G., MEUGNIER H., BES M., VANDENESCH F., ETIENNE J. et TRISTAN A. (2010b). Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible Staphylococcus aureus, 1981-2007. *J Infect Dis*, 2010b, 201, 10, p. 1589-97.

RAUTENBERG M., JOO H. S., OTTO M. et PESCHEL A. (2011). Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. *Faseb J*, 2011, 25, 4, p. 1254-63.

REILLY S. S., HUDSON M. C., KELLAM J. F. et RAMP W. K. (2000). In vivo internalization of *Staphylococcus aureus* by embryonic chick osteoblasts. *Bone*, 2000, 26, 1, p. 63-70.

REOTT M. A., JR., RITCHIE-MILLER S. L., ANGUITA J. et HUDSON M. C. (2008). TRAIL expression is induced in both osteoblasts containing intracellular *Staphylococcus aureus* and uninfected osteoblasts in infected cultures. *FEMS Microbiol Lett*, 2008, 278, 2, p. 185-92.

RIBEIRO A., DIAS C., SILVA-CARVALHO M. C., BERQUO L., FERREIRA F. A., SANTOS R. N., FERREIRA-CARVALHO B. T. et FIGUEIREDO A. M. (2005). First report of infection with community-acquired methicillin-resistant *Staphylococcus aureus* in South America. *J Clin Microbiol*, 2005, 43, 4, p. 1985-8.

ROBINSON D. A. et ENRIGHT M. C. (2003). Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*, 2003, 47, 12, p. 3926-34.

ROBINSON D. A., KEARNS A. M., HOLMES A., MORRISON D., GRUNDMANN H., EDWARDS G., O'BRIEN F. G., TENOVER F. C., MCDOUGAL L. K., MONK A. B. et ENRIGHT M. C. (2005). Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. *Lancet*, 2005, 365, 9466, p. 1256-8.

ROBINSON L. J., BORYSENKO C. W. et BLAIR H. C. (2007). Tumor necrosis factor family receptors regulating bone turnover: new observations in osteoblastic and osteoclastic cell lines. *Ann N Y Acad Sci*, 2007, 1116, p. 432-43.

RYAN M. J., KAVANAGH R., WALL P. G. et HAZLEMAN B. L. (1997). Bacterial joint infections in England and Wales: analysis of bacterial isolates over a four year period. *Br J Rheumatol*, 1997, 36, 3, p. 370-3.

SAEZ-LLORENS X., MUSTAFA M. M., RAMILO O., FINK C., BEUTLER B. et NELSON J. D. (1990). Tumor necrosis factor alpha and interleukin 1 beta in synovial fluid of infants and children with suppurative arthritis. *Am J Dis Child*, 1990, 144, 3, p. 353-6.

SALO J., LEHENKARI P., MULARI M., METSIKKO K. et VAANANEN H. K. (1997). Removal of osteoclast bone resorption products by transcytosis. *Science*, 1997, 276, 5310, p. 270-3.

SCHLIEVERT P. M., STRANDBERG K. L., LIN Y. C., PETERSON M. L. et LEUNG D. Y. (2010). Secreted virulence factor comparison between methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, and its relevance to atopic dermatitis. *J Allergy Clin Immunol*, 2010, 125, 1, p. 39-49.

SCHNAITH A., KASHKAR H., LEGGIO S. A., ADDICKS K., KRONKE M. et KRUT O. (2007). *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. *J Biol Chem*, 2007, 282, 4, p. 2695-706.

SCHWANK S., RAJACIC Z., ZIMMERLI W. et BLASER J. (1998). Impact of bacterial biofilm formation on in vitro and in vivo activities of antibiotics. *Antimicrob Agents Chemother*, 1998, 42, 4, p. 895-8.

SHIRTLIFF M. E. et MADER J. T. (2002). Acute septic arthritis. *Clin Microbiol Rev*, 2002, 15, 4, p. 527-44.

SINHA B., FRANCOIS P. P., NUSSE O., FOTI M., HARTFORD O. M., VAUDAUX P., FOSTER T. J., LEW D. P., HERRMANN M. et KRAUSE K. H. (1999). Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol*, 1999, 1, 2, p. 101-17.

SMITH D. D., GOWEN M. et MUNDY G. R. (1987). Effects of interferon-gamma and other cytokines on collagen synthesis in fetal rat bone cultures. *Endocrinology*, 1987, 120, 6, p. 2494-9.

SONG L., HOBAUGH M. R., SHUSTAK C., CHELEY S., BAYLEY H. et GOUAUX J. E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science*, 1996, 274, 5294, p. 1859-66.

STEFANI S., BONGIORNO D., CAFISO V., CAMPANILE F., CRAPIS M., CRISTINI F., SARTOR A., SCARPARO C., SPINA D. et VIALE P. (2009). Pathotype and susceptibility profile of a community-acquired methicillin-resistant *Staphylococcus aureus* strain responsible for a case of severe pneumonia. *Diagn Microbiol Infect Dis*, 2009, 63, 1, p. 100-4.

STEVENS D. L., MA Y., SALMI D. B., MCINDOO E., WALLACE R. J. et BRYANT A. E. (2007). Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. *J Infect Dis*, 2007, 195, 2, p. 202-11.

STOTT N. S. (2001). Paediatric bone and joint infection. *J Orthop Surg (Hong Kong)*, 2001, 9, 1, p. 83-90.

SUN F., CHO H., JEONG D. W., LI C., HE C. et BAE T. (2010). Aureusimines in *Staphylococcus aureus* are not involved in virulence. *PLoS One*, 2010, 5, 12, p. e15703.

SUREWAARD B. G., NIJLAND R., SPAAN A. N., KRUIJTZER J. A., DE HAAS C. J. et VAN STRIJP J. A. (2012). Inactivation of staphylococcal phenol soluble modulins by serum lipoprotein particles. *PLoS Pathog*, 2012, 8, 3, p. e1002606.

SZMIGIELSKI S., JELJASZEWICZ J., WILCZYNSKI J. et KORBECKI M. (1966). Reaction of rabbit leucocytes to staphylococcal (Panton-Valentine) leucocidin in vivo. *J Pathol Bacteriol*, 1966, 91, 2, p. 599-604.

SZMIGIELSKI S., PREVOST G., MONTEIL H., COLIN D. A. et JELJASZEWICZ J. (1999). Leukocidal toxins of staphylococci. *Zentralbl Bakteriol*, 1999, 289, 2, p. 185-201.

TAKEUCHI O., HOSHINO K., KAWAI T., SANJO H., TAKADA H., OGAWA T., TAKEDA K. et AKIRA S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*, 1999, 11, 4, p. 443-51.

TAUBMAN M. A. et KAWAI T. (2001). Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption. *Crit Rev Oral Biol Med*, 2001, 12, 2, p. 125-35.

THEOLEYRE S., WITTRANT Y., TAT S. K., FORTUN Y., REDINI F. et HEYMANN D. (2004). The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev*, 2004, 15, 6, p. 457-75.

THOENDEL M., KAVANAUGH J. S., FLACK C. E. et HORSWILL A. R. (2011). Peptide signaling in the staphylococci. *Chem Rev*, 2011, 111, 1, p. 117-51.

THOMSON B. M., MUNDY G. R. et CHAMBERS T. J. (1987). Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. *J Immunol*, 1987, 138, 3, p. 775-9.

TING J. P., WILLINGHAM S. B. et BERGSTRALH D. T. (2008). NLRs at the intersection of cell death and immunity. *Nat Rev Immunol*, 2008, 8, 5, p. 372-9.

TOKUKODA Y., TAKATA S., KAJI H., KITAZAWA R., SUGIMOTO T. et CHIHARA K. (2001). Interleukin-1beta stimulates transendothelial mobilization of human peripheral blood mononuclear cells with a potential to differentiate into osteoclasts in the presence of osteoblasts. *Endocr J*, 2001, 48, 4, p. 443-52.

TRAMPUZ A. et ZIMMERLI W. (2008). Diagnosis and treatment of implant-associated septic arthritis and osteomyelitis. *Curr Infect Dis Rep*, 2008, 10, 5, p. 394-403.

TRISTAN A., BES M., MEUGNIER H., LINA G., BOZDOGAN B., COURVALIN P., REVERDY M. E., ENRIGHT M. C., VANDENESCH F. et ETIENNE J. (2007a). Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant *Staphylococcus aureus*, 2006. *Emerg Infect Dis*, 2007a, 13, 4, p. 594-600.

TRISTAN A., FERRY T., DURAND G., DAUWALDER O., BES M., LINA G., VANDENESCH F. et ETIENNE J. (2007b). Virulence determinants in community and hospital meticillin-resistant *Staphylococcus aureus*. *J Hosp Infect*, 2007b, 65 Suppl 2, p. 105-9.

TSEZOU A., POULTSIDES L., KOSTOPOULOU F., ZINTZARAS E., SATRA M., KITSIOU-TZELI S. et MALIZOS K. N. (2008). Influence of interleukin 1alpha (IL-1alpha), IL-4, and IL-6 polymorphisms on genetic susceptibility to chronic osteomyelitis. *Clin Vaccine Immunol*, 2008, 15, 12, p. 1888-90.

TUCHSCHERR L., HEITMANN V., HUSSAIN M., VIEMANN D., ROTH J., VON EIFF C., PETERS G., BECKER K. et LOFFLER B. (2010). *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis*, 2010, 202, 7, p. 1031-40.

TUCKER K. A., REILLY S. S., LESLIE C. S. et HUDSON M. C. (2000). Intracellular *Staphylococcus aureus* induces apoptosis in mouse osteoblasts. *FEMS Microbiol Lett*, 2000, 186, 2, p. 151-6.

U.K. HEALTH PROTECTION AGENCY (2008). Surgical Site Infection - National aggregated data on Surgical Site Infections for hospitals that have participated in Surgical Site Infection Surveillance Scheme (SSISS) between October 1997 and December 2005. http://www.hpa.org.uk/HPA/Topics/InfectiousDiseases/InfectionsAZ/1202115536296/. 2008, p.

UDAGAWA N., TAKAHASHI N., KATAGIRI T., TAMURA T., WADA S., FINDLAY D. M., MARTIN T. J., HIROTA H., TAGA T., KISHIMOTO T. et SUDA T. (1995). Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. *J Exp Med*, 1995, 182, 5, p. 1461-8.

UDO E. E., PEARMAN J. W. et GRUBB W. B. (1993). Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect*, 1993, 25, 2, p. 97-108.

VAANANEN H. K. et LAITALA-LEINONEN T. (2008). Osteoclast lineage and function. *Arch Biochem Biophys*, 2008, 473, 2, p. 132-8.

VALEVA A., WALEV I., PINKERNELL M., WALKER B., BAYLEY H., PALMER M. et BHAKDI S. (1997). Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci U S A*, 1997, 94, 21, p. 11607-11.

VALLENET D., ENGELEN S., MORNICO D., CRUVEILLER S., FLEURY L., LAJUS A., ROUY Z., ROCHE D., SALVIGNOL G., SCARPELLI C. et MEDIGUE C. (2009). MicroScope: a platform for microbial genome annotation and comparative genomics. *Database (Oxford)*, 2009, 2009, p. bap021.

VALLIANOU N., EVANGELOPOULOS A., MAKRI P., ZACHARIAS G., STEFANITSI P., KARACHALIOS A. et AVGERINOS P. C. (2008). Vertebral osteomyelitis and native valve endocarditis due to *Staphylococcus simulans*: a case report. *J Med Case Rep*, 2008, 2, p. 183.

VANDENESCH F., NAIMI T., ENRIGHT M. C., LINA G., NIMMO G. R., HEFFERNAN H., LIASSINE N., BES M., GREENLAND T., REVERDY M. E. et ETIENNE J.

(2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis*, 2003, 9, 8, p. 978-84.

VANDER HAVE K. L., KARMAZYN B., VERMA M., CAIRD M. S., HENSINGER R. N., FARLEY F. A. et LUBICKY J. P. (2009). Community-associated methicillin-resistant *Staphylococcus aureus* in acute musculoskeletal infection in children: a game changer. *J Pediatr Orthop*, 2009, 29, 8, p. 927-31.

VELAZQUEZ-MEZA M. E., AIRES DE SOUSA M., ECHANIZ-AVILES G., SOLORZANO-SANTOS F., MIRANDA-NOVALES G., SILVA-SANCHEZ J. et DE LENCASTRE H. (2004). Surveillance of methicillin-resistant *Staphylococcus aureus* in a pediatric hospital in Mexico City during a 7-year period (1997 to 2003): clonal evolution and impact of infection control. *J Clin Microbiol*, 2004, 42, 8, p. 3877-80.

VON EIFF C., BETTIN D., PROCTOR R. A., ROLAUFFS B., LINDNER N., WINKELMANN W. et PETERS G. (1997). Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clin Infect Dis*, 1997, 25, 5, p. 1250-1.

VON EIFF C., PETERS G. et BECKER K. (2006). The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. *Injury*, 2006, 37 Suppl 2, p. S26-33.

WADA T., NAKASHIMA T., HIROSHI N. et PENNINGER J. M. (2006). RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med*, 2006, 12, 1, p. 17-25.

WAJANT H., PFIZENMAIER K. et SCHEURICH P. (2003). Tumor necrosis factor signaling. *Cell Death Differ*, 2003, 10, 1, p. 45-65.

WALEV I., MARTIN E., JONAS D., MOHAMADZADEH M., MULLER-KLIESER W., KUNZ L. et BHAKDI S. (1993). Staphylococcal alpha-toxin kills human keratinocytes by permeabilizing the plasma membrane for monovalent ions. *Infect Immun*, 1993, 61, 12, p. 4972-9.

WANG B., YURECKO R. S., DEDHAR S. et CLEARY P. P. (2006). Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. *Cell Microbiol*, 2006, 8, 2, p. 257-66.

WANG R., BRAUGHTON K. R., KRETSCHMER D., BACH T. H., QUECK S. Y., LI M., KENNEDY A. D., DORWARD D. W., KLEBANOFF S. J., PESCHEL A., DELEO F. R. et OTTO M. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med*, 2007, 13, 12, p. 1510-4.

WARD P. D. et TURNER W. H. (1980). Identification of staphylococcal Panton-Valentine leukocidin as a potent dermonecrotic toxin. *Infect Immun*, 1980, 28, 2, p. 393-7.

WEICHERT S., SHARLAND M., CLARKE N. M. et FAUST S. N. (2008). Acute haematogenous osteomyelitis in children: is there any evidence for how long we should treat? *Curr Opin Infect Dis*, 2008, 21, 3, p. 258-62.

XING L., CARLSON L., STORY B., TAI Z., KENG P., SIEBENLIST U. et BOYCE B. F. (2003). Expression of either NF-kappaB p50 or p52 in osteoclast precursors is required for IL-1-induced bone resorption. *J Bone Miner Res*, 2003, 18, 2, p. 260-9.

XU K. D., MCFETERS G. A. et STEWART P. S. (2000). Biofilm resistance to antimicrobial agents. *Microbiology*, 2000, 146 (Pt 3), p. 547-9.

YASUDA H., SHIMA N., NAKAGAWA N., YAMAGUCHI K., KINOSAKI M., MOCHIZUKI S., TOMOYASU A., YANO K., GOTO M., MURAKAMI A., TSUDA E., MORINAGA T., HIGASHIO K., UDAGAWA N., TAKAHASHI N. et SUDA T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*, 1998, 95, 7, p. 3597-602.

YOSHII T., MAGARA S., MIYAI D., NISHIMURA H., KUROKI E., FURUDOI S., KOMORI T. et OHBAYASHI C. (2002). Local levels of interleukin-1beta, -4, -6

and tumor necrosis factor alpha in an experimental model of murine osteomyelitis due to staphylococcus aureus. *Cytokine*, 2002, 19, 2, p. 59-65.

YTHIER M., ENTENZA J. M., BILLE J., VANDENESCH F., BES M., MOREILLON P. et SAKWINSKA O. (2010). Natural variability of in vitro adherence to fibrinogen and fibronectin does not correlate with in vivo infectivity of *Staphylococcus aureus*. *Infect Immun*, 2010, 78, 4, p. 1711-6.

ZIMMERLI W., TRAMPUZ A. et OCHSNER P. E. (2004). Prosthetic-joint infections. *N Engl J Med*, 2004, 351, 16, p. 1645-54.

ZOU W., HAKIM I., TSCHOEP K., ENDRES S. et BAR-SHAVIT Z. (2001). Tumor necrosis factor-alpha mediates RANK ligand stimulation of osteoclast differentiation by an autocrine mechanism. *J Cell Biochem*, 2001, 83, 1, p. 70-83.

ANNEXE I

Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981-2007

Rasigade JP, Laurent F, Lina G, Meugnier H, Bes M, Vandenesch F, Etienne E, Tristan A.

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MAJOR ARTICLE

Global Distribution and Evolution of Panton-Valentine Leukocidin–Positive Methicillin-Susceptible *Staphylococcus aureus*, 1981–2007

Jean-Philippe Rasigade, Frederic Laurent, Gerard Lina, Helene Meugnier, Michele Bes, François Vandenesch, Jerome Etienne, and Anne Tristan

Université Lyon 1, Centre National de Référence des Staphylocoques, and Institut National de la Santé et de la Recherche Médicale U851, Institut Fédératif de Recherche 128, Lyon, and Hospices Civils de Lyon, Centre de Biologie Est, Bron, France

Background. Panton-Valentine leukocidin (PVL)–positive methicillin-susceptible *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MSSA and MRSA, respectively) are both associated with severe infections, such as necrotizing pneumonia. The epidemiological profile of PVL-positive community-acquired (CA) MRSA has been extensively studied, but few corresponding data on PVL-positive MSSA are available.

Objectives. The objectives of the study were to investigate the global population structure of PVL-positive MSSA, to compare it with that reported for CA-MRSA, and thus to examine the phylogenetic relationship between these pathogens.

Methods. We determined the *agr* types, multilocus sequence types, and toxin gene profiles of 211 PVL-positive MSSA clinical isolates collected in 19 countries throughout the world between 1981 and 2007.

Results. The predominant lineages of PVL-positive MSSA were *agr*3/ST30, *agr*4/ST121, *agr*3/ST1, *agr*2/ST5, and *agr*3/ST80. Except for *agr*4/ST121, these lineages are also reported to be prevalent among CA-MRSA. PVL-positive MSSA lineages that are genetically related to CA-MRSA have gradually replaced other lineages (especially *agr*4/ST121) over the past 2 decades. Within a given sequence type, the toxin gene content of PVL-positive MSSA strains was very similar to that of PVL-positive CA-MRSA.

Conclusions. The molecular epidemiological profiles of PVL-positive MSSA and CA-MRSA are dynamically interrelated, with the former appearing to constitute a reservoir for the latter.

Staphylococcus aureus is a major human pathogen that causes diseases ranging from minor skin and soft-tissue infections (SSTIs) to life-threatening pneumonia and toxin-mediated diseases, such as toxic shock syndrome [1]. *S. aureus* is the most frequently occurring pathogen in hospitals and the second most common pathogen in outpatient settings [2]. *S. aureus* SSTIs and severe deep-seated infections, such as necrotizing pneumonia, are frequently associated with strains harboring the *lukPV* locus encoding the Panton-Valentine leukocidin (PVL). PVL, a pore-forming protein exotoxin, can recruit polymorphonuclear cells and monocytes and trigger their apoptosis and lysis [3, 4]. Both methicillin-susceptible *S. aureus* (MSSA) and community-acquired methicillin-resistant *S. aureus* (CA-MRSA) can express PVL [5]. It is unclear why *lukPV* is frequently associated with the methicillin resistance locus in community isolates, especially because these transferable elements integrate distinct parts of the genome [4].

Epidemiological studies of PVL-positive *S. aureus* (mainly MRSA) have revealed a highly clonal population structure, and the worrisome recent upsurge in PVL-positive CA-MRSA is due to a limited number of pandemic lineages. The most prevalent genotypes, designated either by their multilocus sequence type or by their pulsotype, are ST80 in Europe, ST8-USA300 in the United States, and ST30 (Southwest Pacific clone),

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Reprints or correspondence: Dr. Jean-Philippe Rasigade, Centre National de Référence des Staphylocoques, Faculté de Médecine Laennec, 7 rue Guillaume Paradin, 69372 Lyon Cedex 8, France (jean-philippe.rasigade@chu-lyon.fr).

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Table 1. Geographic Origins and Isolation Periods of 211 Panton-Valentine Leukocidin–Positive Methicillin-Susceptible *Staphylococcus aureus* Isolates

Isolation period	Isolates, no. (%)	Geographic origin (no. of isolates)
1981–1990	16 (7.6)	France (16)
1991–2000	28 (13.3)	France (26), Guiana (1), and Switzerland (1)
2001–2005	109 (51.7)	China (1), France (47), Guiana (2), New Caledonia (6), Polynesia (12), Fr. West Indies (3), Germany (1), Ivory Coast (2), Mayotte (1), Paraguay (4), Romania (1), Spain (1), Sweden (2), Switzerland (3), the Netherlands (3), Togo (11), Tunisia (2), and US (7)
2006–2007	58 (27.5)	Algeria (7), Czech Republic (2), Germany (1), France (21), Guiana (5), New Caledonia (4), Fr. West Indies (1), Morocco (1), Singapore (1), Turkey (2), and UK (13)

NOTE. Fr., French; UK, United Kingdom; US, United States.

which is ubiquitous [6–8]. Less-prevalent CA-MRSA lineages include ST1-USA400, ST5-USA100, and ST59-USA1000 [6, 9, 10].

PVL-positive MSSA strains appear to belong to more-diverse genetic backgrounds than their methicillin-resistant counterparts [11]. For example, Monecke et al [12], using DNA microarray analysis, found that 30 PVL-positive MSSA strains belonged to 8 different sequence types (STs). Interestingly, some PVL-positive MSSA lineages are closely related to epidemic-associated CA-MRSA lineages. This association raises the possibility that PVL-positive CA-MRSA arises from preexisting MSSA lineages by methicillin resistance gene transfer, although one cannot exclude the transfer of PVL genes in some healthcare-associated MRSA genetic backgrounds. Thus, the success of CA-MRSA as a pathogen could be due not only to its antibiotic resistance but, also, to other factors related to its genetic background. However, epidemiological data on PVL-positive MSSA are sparse, and most relevant studies have involved limited geographic areas, such as a single town or healthcare center, and/or short time frames [12, 13].

The aim of the present study was to investigate the population structure and distribution of PVL-positive MSSA, as well as their evolution over time. The study period covered the emergence and spread of CA-MRSA. A total of 211 PVL-positive MSSA isolates were collected from 19 countries throughout the world over 26 years (from 1981 through 2007). The isolates were characterized by means of multilocus sequence typing (MLST) and *spa* typing, and they were screened for 19 virulence genes. Population diversity was assessed using the Simpson diversity index. The population structure of PVL-positive MSSA was then compared with that reported for CA-MRSA, to assess the phylogenetic relationship between these closely related pathogens.

MATERIALS AND METHODS

Bacterial isolates. A total of 211 PVL-positive MSSA clinical isolates were selected from the strain collection of the French National Reference Center for Staphylococci. These isolates were voluntarily sent to the center, for toxin gene profiling, by microbiologists throughout the world. The selected strains were collected in 19 countries spanning 5 continents (Table 1). The study lasted from 1981 through 2007. The strains were associated with a wide variety of diseases, comprising SSTIs ($n = 85$ [40.3%]), necrotizing pneumonia ($n = 71$ [33.6%]), bone and joint infections ($n = 12$ [5.7%]), other infections ($n = 12$ [5.7%]), and unknown infections ($n = 31$ [14.7%]). Each strain was isolated from a different patient.

DNA extraction,* mecA *testing, and identification of* agr *alleles. Genomic DNA was extracted using a standard procedure [14]. *gyr*A amplification was used to confirm the quality of each DNA extract and the absence of polymerase chain reaction (PCR) inhibitors. *agr* alleles and the absence of the *mec*A methicillin resistance gene were identified by PCR, as described elsewhere [14, 15].

Detection of toxin genes. The isolates were screened by PCR for 19 specific staphylococcal virulence genes, as described elsewhere [14], comprising sequences specific for enterotoxins (*sea-d, seh, sek, sel,* and *sep-r*), the enterotoxin gene cluster *(egc)*, toxic shock syndrome toxin–1 *(tst)*, exfoliative toxins (*eta, etb,* and *etd*), PVL (*luk*S-PV and *luk*F-PV), class F lukM leukocidin (*luk*M), epidermal cell differentiation inhibitor (*edin*A/B/C), and β-hemolysin *(hlb)*. This panel of genes was selected because it represents well-characterized virulence factors that are not uniformly distributed in the *S. aureus* population [14].

MLST. All the isolates were studied by MLST, as described elsewhere [16]. The allelic profiles were determined by sequencing 500-bp internal fragments of 7 housekeeping genes (*arc*C, *aro*E, *glp*F, *gmk, pta, tpi,* and *yqi*L). Nucleotide sequences were entered on the MLST home page [17], where 7 numbers depicting the allelic profile were assigned to define the STs. STs that differed by only one locus were grouped together into clonal complexes (CCs) by use of eBURST software (version 3) [18, 19]. To examine possible changes in ST distribution over time, we studied subsets of isolates collected in France during 4 different periods: 1981–1990 ($n = 16$ isolates), 1991–2000 ($n = 26$), 2001–2005 ($n = 45$), and 2006–2007 ($n = 18$).

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Table 2. **Distribution of Panton-Valentine Leukocidin–Positive Methicillin-Susceptible *Staphylococcus aureus* Isolates, According to *agr* Type, Sequence Type (ST), and *spa* Type**

agr type, ST (CC)	Isolates, no. (%)[a]	*spa*CC[b]	*spa* Type[c]	Isolates, no. (%)[d]	Origin(s) of isolates of each ST
*agr*1					
All	61 (28.9)				
ST25	15 (24.6)	78	t078, t349, t1671, and t3644	10 (66.7)	Algeria, France, Polynesia, Paraguay, and US
		sgl	t528, t643, t1851, and t3640	5 (33.3)	
ST152	13 (21.3)	355	t355, t1096, and t1299	9 (69.2)	France, Fr. West Indies, and Togo
		44	t044[e]	3 (23.1)	
		sgl	t3621	1 (7.7)	
ST188	10 (16.4)	189	t189 and t2612	10 (100.0)	France, New Caledonia, and Polynesia
ST217 (CC22)	6 (9.8)	5/891	t005 and t891	4 (66.7)	France, Sweden, Switzerland, and UK
		sgl	t417 and t3379	2 (33.3)	
ST22 (CC22)	3 (4.9)	5/891	t005	1 (33.3)	France and Turkey
		sgl	t3615 and t3638	2 (66.7)	
ST291	4 (6.6)	1149	t1149, t1599, t3642, and t3649	4 (100.0)	Algeria, France, and UK
ST8	4 (6.6)	8/24	t008 and t024	4 (100.0)	France, Switzerland, and US
ST45	1 (1.6)	sgl	t065	1 (100.0)	Algeria
ST59	1 (1.6)	sgl	t437	1 (100.0)	France
ST398	1 (1.6)	sgl	t571	1 (100.0)	France
ST669	1 (1.6)	44	t359	1 (100.0)	France
ST1280	1 (1.6)	sgl	t3619	1 (100.0)	Guiana
ST1290	1 (1.6)	44	t131[e]	1 (100.0)	UK
*agr*2	18 (8.5)				
ST5	14 (77.8)	2	t1215, t002, t105, t311, and t306	14 (100.0)	France, Guiana, Ivory Coast, Mayotte, Togo, and UK
ST15	3 (16.7)	84/85	t084 and t085	3 (100.0)	Algeria and France
ST1279	1 (5.6)	sgl	t903	1 (100.0)	France
*agr*3	89 (42.2)				
ST30 (CC30)	42 (47.2)	21	t318, t021, t019, t433, t012, t122, t342, t363, t582, t1055, t1382, t1605, t1848, t3620, t3623, t3634, t3641, and t3646	42 (100.0)	France, UK, New Caledonia, Germany, Czech Republic, Morocco, the Netherlands, Tunisia, Guiana, US, Paraguay, Togo, and Sweden
ST37 (CC30)	1 (1.1)	21	t300	1 (100.0)	France
ST1 (CC01)	20 (22.5)	127	t590, t127, t3636, t114, t1849, and t1931	19 (95.0)	Polynesia, Fr. West Indies, Togo, Algeria, UK, New Caledonia, Singapore, France, Ivory Coast, and Guiana
		sgl	t559	1 (5.0)	
ST567 (CC01)	2 (2.2)	127	t1242	2 (100.0)	Polynesia
ST1278 (CC01)	1 (1.1)	sgl	t3643	1 (100.0)	France
ST80	14 (15.7)	44	t044,[e] t131,[e] t376, and t934	14 (100.0)	Algeria, France, and Togo
ST88	6 (6.7)	2393	t2393, t786, t1604, t2742, and t3622	6 (100.0)	New Caledonia, UK, China, and US
ST446	1 (1.1)	sgl	t1710[e]	1 (100.0)	Switzerland
ST776	1 (1.1)	21	t399	1 (100.0)	UK
ST918	1 (1.1)	sgl	t1710[e]	1 (100.0)	France
*agr*4	43 (20.4)				
ST121	42 (97.7)	159	t645,[e] t159, t284, t435, t940, t169, t1077, t1114, t1596, t3407, and t3635	28 (66.7)	Paraguay, New Caledonia, Togo, France, Czech Republic, Germany, Turkey, US, Fr. West Indies, UK, Polynesia, Switzerland, Spain, Algeria, and the Netherlands
		3616	t1850, t3613, t3616, and t3650	4 (9.5)	
		sgl	t314	8 (19.0)	
		sgl	t2304 and t1211	2 (4.8)	
ST946	1 (2.3)	159	t645[e]	1 (100.0)	Romania

NOTE. *agr*, accessory gene regulator; CC, clonal complex; Fr., French; sgl, singleton; *spa*CC, *spa* clonal complex; ST, sequence type; UK, United Kingdom; US, United States.

[a] Data are the no. of isolates of each *agr* type and ST (% of isolates of a given *agr* type in all isolates or % of isolates of a given ST in the isolates of the same *agr* type).

[b] When 2 *spa* types within a given *spa*CC have the same probability of being its founder, the CC is designated by these 2 spa types separated by a virgule.

[c] In order of decreasing frequency.

[d] Data are the no. of isolates of each *spa*CC (% of isolates of the corresponding *spa*CC in the isolates of the same ST).

[e] *spa* type found in ⩾2 STs.

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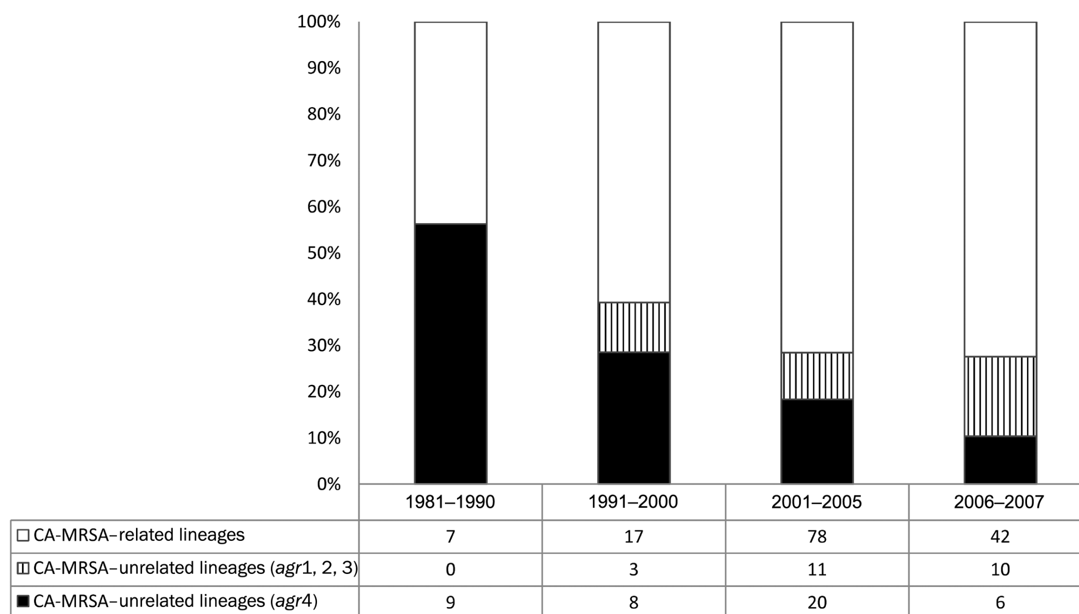

	1981–1990	1991–2000	2001–2005	2006–2007
□ CA-MRSA–related lineages	7	17	78	42
◫ CA-MRSA–unrelated lineages (*agr*1, 2, 3)	0	3	11	10
■ CA-MRSA–unrelated lineages (*agr*4)	9	8	20	6

Figure 1. Distribution of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)–related and –unrelated lineages among Panton-Valentine leukocidin–positive methicillin-susceptible *S. aureus* (MSSA) ($n = 211$), as well as changes over time. CA-MRSA–related lineages were accessory gene regulator *(agr)*1/clonal complex (CC) 22, sequence type (ST) 25, ST152, ST8, ST59, and ST398; *agr*2/ST5; and *agr*3/CC30, CC01, ST80, ST88, and ST776. CA-MRSA–unrelated lineages were mostly *agr*4/CC121, an *agr* group in which no MRSA strains have been described to date. The other CA-MRSA–unrelated lineages were *agr*1/ST188, ST291, ST45, ST669, ST1280, and 1290; *agr*2/ST15 and ST1279; and *agr*3/ST446 and ST918.

This analysis was restricted to French isolates, to limit the bias resulting from variations over time in the inclusion of samples from other countries.

spa *typing.* All the isolates were also *spa*-typed, as described elsewhere [20]. The repeat region of the protein A gene *(spa)* was amplified by PCR and sequenced. *spa* types were determined using Ridom Staph Type software (version 1.5; Ridom), which automatically detects *spa* repeats and assigns a *spa* type according to Harmsen et al [21, 22]. *spa* types were clustered into CCs (ie, *spa*CCs) by use of the integrated BURP (Based Upon Repeat Patterns) algorithm [23, 24]. User-definable parameters were set as follows: "cluster *spa* types into *spa*CC if cost distances are less than or equal to 4" and "exclude *spa* types shorter than 5 repeats." This parameter combination ensures optimal concordance (95.3%) between BURP and e-BURST [25].

Antimicrobial susceptibility testing and detection of remnant SCC*mec *elements in ST80 isolates. Most ST80 CA-MRSA isolates exhibit a particular resistance profile, with resistance to β-lactams, kanamycin, tetracycline, and fusidic acid [6]. We determined the resistance profiles of all ST80 PVL-positive MSSA isolates and searched for similarities between their profile and that of their CA-MRSA counterparts. Susceptibility to penicillin, oxacillin, cefoxitin, kanamycin, tobramycin, gentamicin, erythromycin, clindamycin, tetracycline, pristinamycin, ofloxacin, fusidic acid, vancomycin, teicoplanin, fosfomycin, trimethoprim/sulfamethoxazole, rifampin, mupirocin, quinupristin/dalfopristin, and linezolid was determined with the standard agar diffusion technique, as recommended by the French Society for Microbiology [26].

Because of the similarities observed between PVL-positive ST80 MSSA and CA-MRSA with respect to their susceptibility patterns, we raised the hypothesis that certain ST80 MSSA isolates were descended from ST80 CA-MRSA after SCC*mec* excision. The Infectio Diagnostic Incorporated (IDI)–MRSA PCR assay (BD Geneohm) was applied to all ST80 isolates, as described elsewhere [27, 28]. This assay detects SCC*mec* elements remaining in MSSA after partial excision of SCC*mec*. It uses 5 primers that are specific for the different SCC*mec* sequences located downstream of the *mec*A gene, plus a primer specific for the *S. aureus* chromosomal gene *orfX*. This conserved open-reading frame flanks the SCC*mec* integration site *attB*. SCC*mec*–specific amplicons were detected with 3 molecular beacon probes.

To identify the SCC*mec* type of the remnant SCC*mec* elements, all ST80 PVL-positive MSSA isolates were screened for SCC*mec* markers—namely, *ccrA, ccrB, dcs* region, Δ*mecR, mecR, mecI,* and *ugpQ*—by use of DNA array (CLONDIAG Chip Technologies), as described elsewhere [29].

Population diversity assessment. Genetic diversity was estimated using the modified Simpson index $\lambda = 1 - \Sigma p_i^2$, where p_i is the proportion of isolates belonging to the ith genotype,

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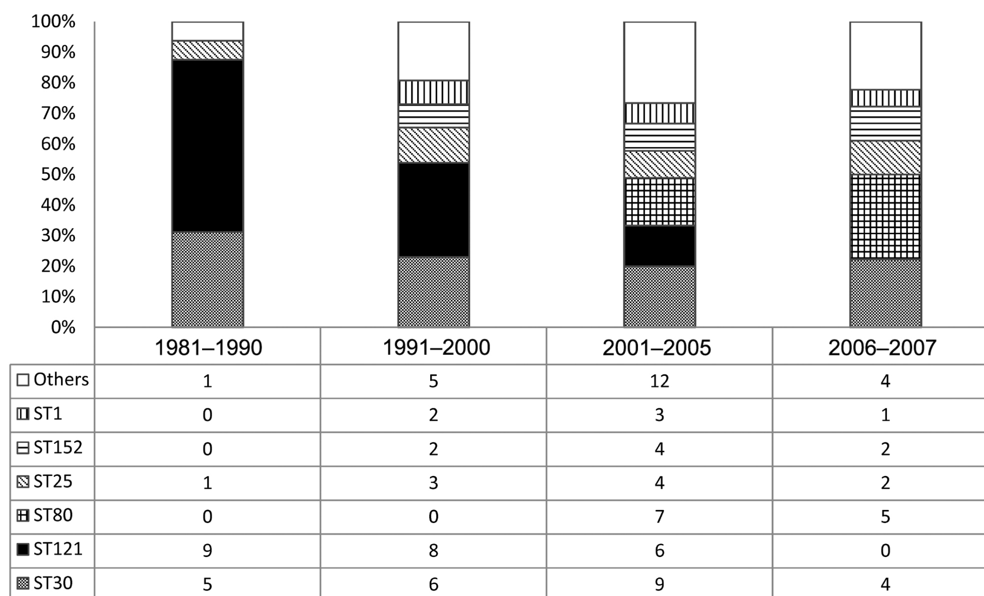

	1981–1990	1991–2000	2001–2005	2006–2007
Others	1	5	12	4
ST1	0	2	3	1
ST152	0	2	4	2
ST25	1	3	4	2
ST80	0	0	7	5
ST121	9	8	6	0
ST30	5	6	9	4

Figure 2. Changes in the distribution of Panton-Valentine leukocidin–positive methicillin-susceptible *Staphylococcus aureus* (MSSA) in France, 1981–2007. Sequence types (STs) designated as "others" were, in order of decreasing frequency, ST5, ST217, ST15, ST188, ST291, ST8, ST22, ST37, ST59, ST398, ST669, ST918, ST1278, and ST1279.

as defined by MLST [30, 31]. The λ value can be described as the probability that 2 isolates chosen at random will have different genotypes, and it thus can be expressed as a percentage [32]. λ increases with the number of genotypes and, also, with more-balanced genotype distributions. Approximate 95% confidence intervals (CIs) were calculated as described elsewhere [32]. The λ value was calculated for the PVL-positive MSSA population, by comparison with (1) a set of CA-MRSA strains characterized by the French National Reference Center for Staphylococci [6] and selected for their similar clinical and geographic origins, and (2) the global *S. aureus* population, based on the MLST database [17] available online on 22 January 2009 (which included 2314 isolates belonging to 919 different STs). To optimize the comparison of PVL-positive MSSA and CA-MRSA, diversity was calculated for a subset of 118 PVL-positive MSSA isolates collected during the same period as the CA-MRSA panel (1999 through 2005).

RESULTS

agr *types, STs, and CCs.* The 211 PVL-positive MSSA isolates were *agr*1 (*n* = 61 [28.9%]), *agr*2 (*n* = 18 [8.5%]), *agr*3 (*n* = 89 [42.2%]), or *agr*4 (*n* = 43 [20.4%]) (Table 2). They belonged to 28 STs. eBURST analysis identified 3 CCs—namely, CC1 (ST1, ST567, and ST1278), CC22 (ST22 and ST217), and CC30 (ST30 and ST37). The remaining 21 STs were singletons. The most frequent STs were ST30 (*n* = 42 [19.9%]), ST121 (*n* = 42 [19.9%]), ST1 (*n* = 20 [9.5%]), ST25 (*n* = 15 [7.1%]), ST5 (*n* = 14 [6.6%]), and ST80 (*n* = 14 [6.6%]). The STs were strictly specific for *agr* types.

Twelve (42.9%) of the 28 STs—namely, ST1 (CC1), ST5, ST8, ST22 (CC22), ST25, ST30 (CC30), ST37 (CC30), ST59, ST80, ST88, ST152, and ST398—had been attributed to CA-MRSA in other studies, whereas 4 other STs—namely, ST567 (CC1), ST1278 (CC1), ST217 (CC22), and ST776 (CC30)—belonged to CA-MRSA CCs [6, 7, 9, 33–43]. Thus, 16 STs (57.1%) were shared by PVL-positive MSSA and CA-MRSA. To our knowledge, no CA-MRSA belonging to the other 12 STs (42.9%; ie, ST15, ST45, ST121, ST188, ST291, ST446, ST669, ST918, ST946, ST1279, ST1280, and ST1290) have been identified to date. These latter 12 STs accounted for 67 (31.8%) of the 211 PVL-positive MSSA isolates, and nearly two-thirds of these isolates (43 [64.2%]) had the *agr*4 (ST121 and ST946) genetic background. The proportion of PVL-positive MSSA isolates that were unrelated to CA-MRSA decreased from 56.3% before 1990 to 27.6% after 2005 (Figure 1).

The ST distribution of the French PVL-positive MSSA isolates changed drastically from 1981 through 2007 (Figure 2). ST80 strains, none of which were found before 2000, accounted for 15.6% of isolates collected during 2001–2005, and ST80 became the most frequent ST (27.8%) identified during 2006–2007. Conversely, ST121 accounted for 56.3% of isolates collected before 1990 but for only 30.8% of isolates in 1991–2000, 13.3% in 2001–2005, and 0% after 2005.

spa *types and* spa*CCs.* The 211 isolates belonged to 96 different *spa* types, corresponding to 14 *spa*CCs and 13 sin-

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Table 3. Toxin Gene Content of Dominant Lineages of Panton-Valentine Leukocidin–Positive Methicillin-Susceptible *Staphylococcus aureus*

ST	Isolates, no. (%)	Toxin gene content, %																	
		sea	*seb*	*sec*	*sed*	*seh*	*sek*	*sel*	*sep*	*seq*	*ser*	*egc*	*tst*	*eta*	*etb*	*etd*	*lukM*	*edin*	*hlb*
ST30	42 (19.9)	26.2	2.4	7.1	…	…	4.8	7.1	…	4.8	…	100.0	14.3	2.4	…	…	…	…	4.8
ST121	42 (19.9)	…	42.9	…	…	…	4.8	…	…	4.8	…	100.0	…	…	2.4	…	…	2.4	2.4
ST1	20 (9.5)	70.0	5.0	…	…	95.0	75.0	…	…	75.0	…	…	…	…	…	…	…	…	5.0
ST25	15 (7.1)	…	60.0	20.0	…	6.7	…	20.0	…	6.7	…	100.0	…	…	…	93.3	…	93.3	6.7
ST5	14 (6.6)	28.6	…	7.1	7.1	…	…	7.1	7.1	…	7.1	100.0	7.1	…	…	…	…	42.9	7.1
ST80	14 (6.6)	…	…	…	…	…	…	…	…	…	7.1	…	…	…	…	100.0	…	100.0	…
ST152	13 (6.2)	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	84.6	84.6
ST188	10 (4.7)	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Other	41 (19.4)	17.1	14.6	7.3	2.4	2.4	17.1	9.8	7.3	17.1	2.4	39.0	4.9	…	…	9.8	…	14.6	9.8
Total	211 (100.0)	17.1	16.6	4.7	0.9	10.0	12.3	5.2	1.9	12.8	1.4	61.1	4.3	0.5	0.5	15.2	…	24.6	10.0

NOTE. …, 0.0%; *edin,* epidermal cell differentiation inhibitor; *egc*, enterotoxin gene cluster; *eta, etb* and *etd,* exfoliative toxin A, B and D, respectively; *lukM,* leukocidin M; *sea* to *sed, seh, sek, sel, sep,* to *ser,* staphylococcal enterotoxin A to D, H, K, L, M, O, and P to R, respectively; ST, sequence type; *tst,* toxic shock toxin.

gletons (14% of *spa* types) (Table 2). Seven *spa* types (8% of *spa* types; 3.8% of all isolates) were excluded from BURP analysis because they consisted of <5 repeats. The most frequent *spa*CCs were *spa*CC21 (MLST CC30) ($n = 44$ [20.9%]), *spa*CC159 (mostly ST121) ($n = 29$ [13.7%]), *spa*CC127 (MLST CC01) ($n = 21$ [10.0%]), and *spa*CC44 (mostly ST80) ($n = 19$ [9.0%]). Of 15 STs comprising >1 isolate, 14 encompassed >1 *spa* type. *spa* types and *spa*CCs were mostly specific for *agr* types and STs. Four *spa* types (t044, t131, t645, and t1710) were found in >1 ST. t645 (*agr*4/ST121 and *agr*4/ST946) and t1710 (*agr*3/ST446 and *agr*3/ST918) were present in STs that were not totally independent (double-locus variants). In contrast, t044 (*agr*3/ST80 and *agr*1/ST152) and t131 (*agr*3/ST80 and *agr*1/ST1290) were present in totally unrelated lineages.

Geographic distribution. Most of the dominant lineages were widely distributed (Table 2). ST30, ST121, and ST1 were pandemic. The most prevalent lineages were, in decreasing frequency, ST30, ST121, and ST80 in Europe and North Africa; ST121, ST5, and ST152 in sub-Saharan Africa; ST121, ST30, and ST25 in North America; and ST30, ST5, ST25, and ST1 in South America.

Toxin genes. Most isolates ($n = 193$ [91.5%]) carried ⩾1 virulence factor other than *pvl* (Table 3). ST188, ST398, ST1279, and ST1280 were the only lineages that did not carry any of the toxin genes sought, apart from *pvl.* The most prevalent toxin locus in PVL-positive MSSA, *egc,* was carried by 61.1% of isolates. *egc* was specific for certain lineages: all isolates belonging to CC30, CC121, ST5, ST25, and CC22 carried *egc,* whereas no isolates belonging to CC1, ST80, ST152, ST188, and ST8 carried *egc.* All ST80 isolates carried *etd* and *edin* and no other toxin genes, except for 1 isolate that also carried *ser.* Apart from *egc,* which is highly prevalent in the general *S. aureus* population [44], the prevalence of the toxin genes ranged from 0.5% (*eta* and *etb*) to 24.6% *(edin).*

Antimicrobial resistance and SCC*mec *remnants in ST80 MSSA isolates. Of the 14 ST80 MSSA isolates, 6 (42.9%) were resistant to penicillin, 1 (7.1%) was resistant to kanamycin, 2 (14.3%) were resistant to erythromycin, 8 (57.1%) were resistant to tetracycline, and 6 (42.9%) were resistant to fusidic acid. The most common phenotype (4 strains [28.6%]) included resistance to penicillin, tetracycline, and fusidic acid. This phenotype resembles that of most ST80 CA-MRSA strains, which are resistant to β-lactams, kanamycin, tetracycline, and fusidic acid [6].

Two ST80 PVL-positive MSSA isolates (14.3%) were positive in the IDI-MRSA assay and carried remnant SCC*mec* elements. DNA array–based characterization of these strains showed that 1 strain carried the *dcs* region, *ccrA-2,* and *ccrB-2,* whereas the other strain carried only the *dcs* region. The *dcs* region is found in SCC*mec* types I, II, and IV. *ccrA-2* and *ccrB-2* are found in SCC*mec* types II and IV [29]. It has been postulated that such strains carrying SCC*mec* remnants may have arisen from MRSA having undergone partial SCC*mec* excision [45]. Each strain was resistant to only one antibiotic class (erythromycin and kanamycin, respectively).

Population diversity. The Simpson diversity index was high for PVL-positive MSSA, with $\lambda = 88.9\%$ (95% CI, 86.8%–90.9%) for the 1981–2007 collection and $\lambda = 89.2\%$ (95% CI, 86.8%–91.7%) for the 1999–2005 collection. On the basis of published data, the index was much lower ($\lambda = 41.0\%$ [95% CI, 35.4%–46.7%]) for the 1999–2005 CA-MRSA collection [6]. The index calculated for the entire *S. aureus* MLST database was $\lambda = 98.0\%$ (95% CI, 97.8%–98.2%).

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DISCUSSION

Several interesting findings emerge from this study of 211 PVL-positive MSSA strains isolated on 5 continents over nearly 3 decades. First, PVL-positive MSSA was more diverse than CA-MRSA: the modified Simpson diversity index λ value for PVL-positive MSSA (89.2%) was 2.2 times higher than that calculated for CA-MRSA (41.0%). This finding indicates that PVL-encoding phages are able to integrate a wide range of unrelated genetic backgrounds. Second, with the exception of ST8, the predominant CA-MRSA lineages reported elsewhere (ST80, ST30, ST8, ST1, and ST5 [6, 7]) were all represented among the predominant lineages of PVL-positive MSSA observed here. Third, among the PVL-positive MSSA lineages, those that were genetically related to CA-MRSA have gradually replaced those unrelated to CA-MRSA (especially *agr*4/ST121) over the past 2 decades. These findings suggest that the molecular epidemiologies of PVL-positive MSSA and CA-MRSA are dynamically interrelated, with the former acting as a reservoir for the latter.

Several predominant genetic backgrounds were found in our collection of PVL-positive MSSA. *agr*3/ST30 and *agr*4/ST121 were found to be pandemic, and each accounted for 19.9% of the PVL-positive MSSA isolates (Table 2). Among the other prevalent lineages, ST1, ST5, ST25, and ST152 were also pandemic, whereas ST80 was found only in Europe and Africa, and ST188 was found only in France, New Caledonia, and Polynesia. However, the observed distribution of PVL-positive MSSA is not fully representative of the current global epidemiology, because of the long inclusion period and the sources of the isolates received by the French National Reference Center for Staphylococci. The low prevalence of ST8 strains in our study might be related to the limited number of strains collected from the United States.

A global analysis of the whole *S. aureus* MLST database showed that MRSA strains were only represented among 215 (23.4%) of the 919 STs. This finding is consistent with the report by Enright et al [46], who found that MRSA strains were represented in only 38 (23.5%) of the 162 *S. aureus* genetic backgrounds described in 2002. This suggests that only a few *S. aureus* lineages are capable of acquiring SCC*mec* [46]. In contrast, most of the PVL-positive MSSA lineages studied here were genetically related to CA-MRSA (16 [57.1%] of 28 STs). We can thus speculate that PVL phages integrate preferentially into SCC*mec*-capable lineages. Further investigations are needed to address this question.

Most of the PVL-positive MSSA strains studied here that were not genetically related to CA-MRSA had the *agr*4 background. As far as we know, this background has never been reported in MRSA and thus seems to be incompatible with stable integration of SCC*mec* elements.

ST80 is the main PVL-positive CA-MRSA lineage in Europe, accounting for 76.1% of CA-MRSA isolates sent to the French National Reference Center for Staphylococci from 1999 through 2005 [6]. It started to emerge in the early 2000s. We found that ST80 PVL-positive MSSA emerged and spread during the same period: ST80 PVL-positive MSSA started to emerge in France between 2001 and 2005, and it was the most frequent PVL-positive MSSA lineage after 2005. The *spa* types, non–β-lactam resistance profiles, and toxin gene content of ST80 PVL-positive MSSA were highly analogous to those of ST80 PVL-positive MRSA [6]. Moreover, 14.3% of ST80 MSSA isolates carried SCC*mec* remnants, indicating that they may correspond to former CA-MRSA strains that have undergone partial SCC*mec* excision. Together, these findings suggest that ST80 PVL-positive *S. aureus* is a successful lineage in which SCC*mec* acquisition and excision have occurred several times.

spa typing with BURP clustering can be used to group *S. aureus* isolates into clonal complexes and to infer their phylogenetic relationships. The inferred phylogeny is consistent with that obtained by MLST and eBURST clustering, with the concordance between these two methods being 95% [25]. However, the same *spa* sequence is sometimes found among phylogenetically unrelated lineages [47], possibly because of repeated convergent evolution (homoplasy) of *spa* sequences rather than because of recombination events. The *spa* gene product, protein A, is a secreted virulence factor involved in host immune system evasion [48]. Because homoplasy results from evolutionary convergence toward the best-adapted phenotype in response to a particular selection pressure, homoplastic *spa* sequences are likely to confer an evolutionary benefit on their host. In the present study, 2 spa types (t044 and t131) appeared to be homoplastic, because they were each found in 2 independent lineages (t044 in *agr*3/ST80 and *agr*1/ST152, and t131 in *agr*3/ST80 and *agr*1/ST1290). Interestingly, these closely related *spa* types are the most prevalent *spa* types in ST80 PVL-positive MSSA and CA-MRSA (Table 2) [6]. The putative evolutionary benefit associated with such *spa* loci might contribute to the success of this lineage. Additional studies are needed to determine whether homoplastic *spa* loci are associated with increased fitness or infectivity.

In conclusion, we found that most predominant genetic backgrounds of PVL-positive MSSA are pandemic and are phylogenetically related to CA-MRSA. In France, the historical ST121 PVL-positive MSSA genetic background has been gradually replaced over the past decade by MSSA lineages related to CA-MRSA (mostly ST80 PVL-positive MSSA). Because PVL-positive MSSA is still the most common cause of severe PVL-associated infections in many countries and is also the most plausible reservoir of CA-MRSA, epidemiological surveillance and infection control efforts must be reinforced.

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References

1. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med **1998**; 339: 520–32.
2. Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E. Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet **2006**; 368:874–85.
3. Kaneko J, Kamio Y. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem **2004**; 68:981–1003.
4. Boyle-Vavra S, Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. Lab Invest **2007**; 87:3–9.
5. Lina G, Piemont Y, Godail-Gamot F, et al. Involvement of Panton-Valentine leukocidin–producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis **1999**; 29:1128–32.
6. Tristan A, Bes M, Meugnier H, et al. Global distribution of Panton-Valentine leukocidin–positive methicillin-resistant *Staphylococcus aureus,* 2006. Emerg Infect Dis **2007**; 13:594–600.
7. Vandenesch F, Naimi T, Enright MC, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis **2003**; 9:978–84.
8. Moran GJ, Krishnadasan A, Gorwitz RJ, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **2006**; 355:666–74.
9. Witte W, Strommenger B, Cuny C, Heuck D, Nuebel U. Methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006. J Antimicrob Chemother **2007**; 60:1258–63.
10. Bartels MD, Boye K, Rhod Larsen A, Skov R, Westh H. Rapid increase of genetically diverse methicillin-resistant *Staphylococcus aureus,* Copenhagen, Denmark. Emerg Infect Dis **2007**; 13:1533–40.
11. Chini V, Petinaki E, Foka A, Paratiras S, Dimitracopoulos G, Spiliopoulou I. Spread of *Staphylococcus aureus* clinical isolates carrying Panton-Valentine leukocidin genes during a 3-year period in Greece. Clin Microbiol Infect **2006**; 12:29–34.
12. Monecke S, Slickers P, Ellington MJ, Kearns AM, Ehricht R. High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus.* Clin Microbiol Infect **2007**; 13:1157–64.
13. McCaskill ML, Mason EO Jr, Kaplan SL, Hammerman W, Lamberth LB, Hulten KG. Increase of the USA300 clone among community-acquired methicillin-susceptible *Staphylococcus aureus* causing invasive infections. Pediatr Infect Dis J **2007**; 26:1122–7.
14. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect Immun **2002**; 70:631–41.
15. Garnier F, Tristan A, Francois B, et al. Pneumonia and new methicillin-resistant *Staphylococcus aureus* clone. Emerg Infect Dis **2006**; 12:498–500.
16. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus.* J Clin Microbiol **2000**; 38:1008–15.
17. Multi Locus Sequence Typing. http://saureus.mlst.net. Accessed 15 March 2010.
18. eBURSTv3. http://eburst.mlst.net. Accessed 15 March 2010.
19. Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ. Displaying the relatedness among isolates of bacterial species—the eBURST approach. FEMS Microbiol Lett **2004**; 241:129–34.
20. Mellmann A, Friedrich AW, Rosenkotter N, et al. Automated DNA sequence-based early warning system for the detection of methicillin-resistant *Staphylococ cus aureus* outbreaks. PLoS Med **2006**; 3:e33.
21. Harmsen D, Claus H, Witte W, Rothganger J, Turnwald D, Vogel U. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J Clin Microbiol **2003**; 41:5442–8.
22. Ridom SpaServer. http://spaserver.ridom.de. Accessed 15 March 2010.
23. Mellmann A, Weniger T, Berssenbrugge C, et al. Characterization of clonal relatedness among the natural population of *Staphylococcus aureus* strains by using *spa* sequence typing and the BURP (based upon repeat patterns) algorithm. J Clin Microbiol **2008**; 46:2805–8.
24. Sammeth M, Stoye J. Comparing tandem repeats with duplications and excisions of variable degree. IEEE/ACM Trans Comput Biol Bioinform **2006**; 3:395–407.
25. Mellmann A, Weniger T, Berssenbrugge C, et al. Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. BMC Microbiol **2007**; 7:98.
26. French Society for Microbiology. Recommandations du Comite de l'Antibiogramme de la Societe Francaise de Microbiologie. http://www.sfm.asso.fr/doc/download.php?doc = DiU8C&fic = casfm_2009.pdf. Accessed 22 February 2009.
27. Huletsky A, Giroux R, Rossbach V, et al. New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. J Clin Microbiol **2004**; 42:1875–84.
28. Huletsky A, Lebel P, Picard FJ, et al. Identification of methicillin-resistant *Staphylococcus aureus* carriage in less than 1 hour during a hospital surveillance program. Clin Infect Dis **2005**; 40:976–81.
29. Monecke S, Slickers P, Ehricht R. Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol **2008**; 53:237–51.
30. Simpson EH. Measurement of diversity. Nature **1949**; 163:688.
31. Magurran AE. Biological diversity. Curr Biol **2005**; 15:R116–8.
32. Grundmann H, Hori S, Tanner G. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. J Clin Microbiol **2001**; 39:4190–2.
33. Monecke S, Berger-Bachi B, Coombs G, et al. Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. Clin Microbiol Infect **2007**; 13:236–49.
34. Sam IC, Kahar-Bador M, Chan YF, Loong SK, Mohd Nor Ghazali F. Multisensitive community-acquired methicillin-resistant *Staphylococcus aureus* infections in Malaysia. Diagn Microbiol Infect Dis **2008**; 62: 437–9.
35. Yu F, Chen Z, Liu C, et al. Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin Microbiol Infect **2008**; 14:381–4.
36. Denis O, Deplano A, De Beenhouwer H, et al. Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium. J Antimicrob Chemother **2005**; 56:1103–6.
37. Harbarth S, Francois P, Shrenzel J, et al. Community-associated methicillin-resistant *Staphylococcus aureus,* Switzerland. Emerg Infect Dis **2005**; 11:962–5.
38. Maier J, Melzl H, Reischl U, et al. Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* in Germany associated with

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travel or foreign family origin. Eur J Clin Microbiol Infect Dis **2005**; 24:637–9.
39. Ramdani-Bouguessa N, Bes M, Meugnier H, et al. Detection of methicillin-resistant *Staphylococcus aureus* strains resistant to multiple antibiotics and carrying the Panton-Valentine leukocidin genes in an Algiers hospital. Antimicrob Agents Chemother **2006**; 50:1083–5.
40. Takano T, Higuchi W, Otsuka T, et al. Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* strains belonging to multilocus sequence type 59 in Taiwan. Antimicrob Agents Chemother **2008**; 52:837–45.
41. Takizawa Y, Taneike I, Nakagawa S, et al. A Panton-Valentine leucocidin (PVL)-positive community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) strain, another such strain carrying a multiple-drug resistance plasmid, and other more-typical PVL-negative MRSA strains found in Japan. J Clin Microbiol **2005**; 43:3356–63.
42. Yamamoto T, Dohmae S, Saito K, et al. Molecular characteristics and in vitro susceptibility to antimicrobial agents, including the des-fluoro(6) quinolone DX-619, of Panton-Valentine leucocidin-positive methicillin-resistant *Staphylococcus aureus* isolates from the community and hospitals. Antimicrob Agents Chemother **2006**; 50:4077–86.
43. Nulens E, Stobberingh EE, Smeets E, et al. Genetic diversity of methicillin-resistant *Staphylococcus aureus* in a tertiary hospital in The Netherlands between 2002 and 2006. Eur J Clin Microbiol Infect Dis **2009**; 28:631–9.
44. Jarraud S, Peyrat MA, Lim A, et al. *egc,* a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus.* J Immunol **2001**; 166:669–77.
45. Donnio PY, Fevrier F, Bifani P, et al. Molecular and epidemiological evidence for spread of multiresistant methicillin-susceptible *Staphylococcus aureus* strains in hospitals. Antimicrob Agents Chemother **2007**; 51: 4342–50.
46. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci U S A **2002**; 99:7687–92.
47. Nubel U, Roumagnac P, Feldkamp M, et al. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus.* Proc Natl Acad Sci U S A **2008**; 105:14,130–5.
48. Foster TJ. Immune evasion by staphylococci. Nat Rev Microbiol **2005**;3: 948–58.

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ANNEXE II

Lethal necrotizing pneumonia due to an ST398 *Staphylococcus aureus* strain producing the Panton-Valentine leukocidin

Rasigade JP, Laurent F, Hubert P, Vandenesch F, Etienne J

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Lethal Necrotizing Pneumonia Caused by an ST398 *Staphylococcus aureus* Strain

To the Editor: Several recent studies have shown massive colonization of livestock (especially pigs) and livestock workers by methicillin-resistant *Staphylococcus aureus* (MRSA) in western Europe, Canada, and the United States (*1,2*). Livestock MRSA isolates belong almost exclusively to a single sequence type, ST398. Evidence of zoonotic and interhuman transmission of methicillin-resistant and methicillin-susceptible variants of this hitherto unusual sequence type was recently reported (*1,3*). *S. aureus* ST398 infections in humans with or without a history of contact with livestock include bacteremia, endocarditis, ventilator-associated pneumonia, and wound infections, none of which involve the expression of specific toxins. Indeed, ST398 isolates are negative for all major virulence factors, with the exception of some rare isolates that harbor the genes that encode the Panton-Valentine leukocidin (PVL) (*1*), a toxin that is usually associated with community-acquired MRSA (*4*). We report a case of lethal necrotizing pneumonia caused by a PVL-positive methicillin-susceptible ST398 *S. aureus* isolate.

A previously healthy 14-year-old girl came to the emergency room with influenza-like illness, cough, fever, and a 2-day history of severe abdominal pain. She was given intravenous antibacterial chemotherapy with cefotaxime and amikacin. An exploratory laparotomy showed no signs of abdominal disease. Immediately after surgery, acute respiratory distress syndrome with hemodynamic instability developed in the patient; mechanical ventilation and inotropic support were required. A chest radiograph showed bilateral pulmonary infiltrates and pleural effusion. *S. aureus* was isolated by bronchoalveolar lavage fluid and blood culture, and staphylococcal necrotizing pneumonia was diagnosed. Clinical features, including the preceding influenza-like illness, were highly consistent with those previously reported (*5*). However, viral cultures and immunofluorescence assays were negative for all common respiratory viruses, and, although the patient had positive serologic test results for influenza B virus, antibody titers were too low to affirm influenza B infection. Severity factors were present (*5*), including leukopenia, airway bleeding, and multiorgan failure. She died 6 days after symptom onset, with refractory shock and respiratory failure caused by bilateral pneumothorax. The *S. aureus* strain, which was susceptible to all tested antimicrobial agents except macrolides, was *agr*1/ST398, *spa*-type t571 and nontypeable by *Sma*I pulsed-field gel electrophoresis, which showed its relatedness to livestock-associated strains. The origin of the infection could not be determined. The presence of the genes encoding PVL was confirmed by PCR.

Thus, the spread of *S. aureus* ST398 among livestock is a matter of increasing concern because strains of this sequence type were able to acquire PVL genes and cause necrotizing pneumonia in a young immunocompetent patient. Transmission control and surveillance efforts are urgently needed to prevent further spread of such strains.

Jean-Philippe Rasigade, Frederic Laurent, Philippe Hubert, François Vandenesch, and Jerome Etienne

Author affiliations: Hospices Civils de Lyon, Lyon, France (J.P. Rasigade, F. Laurent, J. Etienne, F. Vandenesch); and Assistance Publique–Hôpitaux de Paris, Paris, France (P. Hubert)

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References

1. Wulf M, Voss A. MRSA in livestock animals—an epidemic waiting to happen? Clin Microbiol Infect. 2008;14:519–21. DOI: 10.1111/j.1469-0691.2008.01970.x
2. Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, Moritz ED, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One. 2009;4:e4258. DOI: 10.1371/journal.pone.0004258
3. Fanoy E, Helmhout LC, van der Vaart WL, Weijdema K, van Santen-Verheuvel MG, Thijsen SF, et al. An outbreak of non-typeable MRSA within a residential care facility. Euro Surveill. 2009;14(1):pii=19080.
4. Chambers HF. Community-associated MRSA-resistance and virulence converge. N Engl J Med. 2005;352:1485–7. DOI: 10.1056/NEJMe058023
5. Gillet Y, Vanhems P, Lina G, Bes M, Vandenesch F, Floret D, et al. Factors predicting mortality in necrotizing community-acquired pneumonia caused by *Staphylococcus aureus* containing Panton-Valentine leukocidin. Clin Infect Dis. 2007;45:315–21. DOI: 10.1086/519263

Address for correspondence: Jean-Philippe Rasigade, Centre de Référence des Staphylocoques, Centre de Biologie Est, Groupement Hospitalier Est, 59 boulevard Pinel, Bron, 69677 France; email: jean-philippe.rasigade@chu-lyon.fr

Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

ANNEXE III

A history of Panton-Valentine leukocidin (PVL)-associated infection protects against death in PVL-associated pneumonia

Rasigade JP, Sicot N, Laurent F, Lina G, Vandenesch F, Etienne J

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Short communication

A history of Panton-Valentine leukocidin (PVL)-associated infection protects against death in PVL-associated pneumonia

Jean-Philippe Rasigade*, Nicolas Sicot, Frederic Laurent, Gerard Lina, François Vandenesch, Jerome Etienne

INSERM U851, National Reference Center for Staphylococci, University of Lyon, Lyon, France

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ABSTRACT

Panton-Valentine leukocidin (PVL) is a *Staphylococcus aureus* toxin associated with skin and soft-tissue infections (SSTIs) and life-threatening necrotizing pneumonia in humans. Recent reports have demonstrated that neutralizing antibody to PVL is not protective against SSTI recurrence, thus raising a controversy about the expected clinical benefits from the use of PVL as a vaccine target. To investigate the impact of pre-existing immunity to PVL on the outcome of necrotizing pneumonia, we conducted a retrospective study of 114 cases and searched for an association between the history of PVL-associated infection and outcome. Death and severity factors, such as the need for mechanical ventilation and inotrope support, were significantly less frequent in patients with prior PVL-associated infection than in those without. These findings indicate a protective role of PVL-directed immunity in severe systemic PVL-associated disease, suggesting that anti-PVL vaccine could provide strong clinical benefits in this setting.

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1. Introduction

The emergence and spread of methicillin-resistant *Staphylococcus aureus* strains producing Panton-Valentine leukocidin (PVL) has become a public health concern in several countries [1,2]. The role of PVL itself in the pathogenesis of infection, which has been initially a matter of debate, is now clearly established and a component of this toxin has been included as a part of a multivalent vaccine under phase-1 clinical trial [3,4]. However, the clinical benefits expected from PVL-directed vaccine strategy remain controversial. Indeed, neutralizing antibody to PVL, besides not being protective against the recurrence of PVL-associated skin and soft-tissue infection (SSTI) in humans [5], also enhanced host susceptibility to infection in a murine abscess model [6]. However, little is known about the influence of pre-existing immunity to PVL in humans in the context of more invasive, life-threatening PVL-associated infections, such as necrotizing pneumonia. To address this question, we conducted a retrospective cohort study of 114 cases of PVL-associated necrotizing pneumonia and searched for an association between a history of PVL-associated infection and the outcome.

2. Patients and methods

Since 1986, reports of *S. aureus* necrotizing pneumonia have been collected by the French National Reference Center for Staphylococci. All cases were spontaneously referred by hospitals in France and French Overseas Departments and Territories. A standardized data form was filled out by the referring clinician of each patient. A history of furuncle was chosen as a marker for prior PVL-associated infection in the patient, because most of the *S. aureus* strains causing furuncles produce PVL [7], whereas PVL prevalence in *S. aureus* from carriage or non-hair follicle-related infections in France is just <2% (J. Etienne, unpublished data). A total of 114 cases of PVL-associated necrotizing pneumonia, in which the clinical and biological data were complete, were included. Clinical features, including 30-day survival and the need for mechanical ventilation and inotrope support, were compared in patients with and without a personal history of furuncle, using a two-sided Fisher's exact test. Multivariate analysis was not performed due to the small number of patients with a history of furuncle.

3. Results and discussion

All patients with a history of furuncle survived, while 35.9% of the patients without a history of furuncle died within 30 days from

* Corresponding author at: INSERM U851, Centre National de Référence des Staphylocoques, Faculté de Médecine Lyon-Est, 7 rue Guillaume Paradin, 69372 Lyon Cedex 8, France. Tel.: +33 0478 77 86 57; fax: +33 0478 77 86 58.
E-mail addresses: jean-philippe.rasigade@chu-lyon.fr, jprasigade@yahoo.fr (J.-P. Rasigade), sicotnicolas@hotmail.com (N. Sicot), frederic.laurent@univ-lyon1.fr (F. Laurent), gerard.lina@univ-lyon1.fr (G. Lina), francois.vandenesch@univ-lyon1.fr (F. Vandenesch), jerome.etienne@univ-lyon1.fr (J. Etienne).

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Table 1
Outcome of Panton-Valentine leukocidin-associated necrotizing pneumonia in 114 patients with and without a history of furuncle.

Evolution and clinical features	No. of patients (n = 114)		p value[a]	Odds ratio (95% CI)
	With a history of furuncle (n = 11)	Without a history of furuncle (n = 103)		
Death within 30 days of hospital admission	0 (0.0%)	37 (35.9%)	0.015	0.00 NC[b]
Mechanical ventilation	3 (27.3%)	67 (65.0%)	0.021	0.20 (0.05–0.81)
Inotrope support	2 (18.2%)	56 (54.4%)	0.028	0.19 (0.04–0.91)

[a] Differences between the groups were tested for statistical significance using Fisher's exact test.
[b] NC, not calculable.

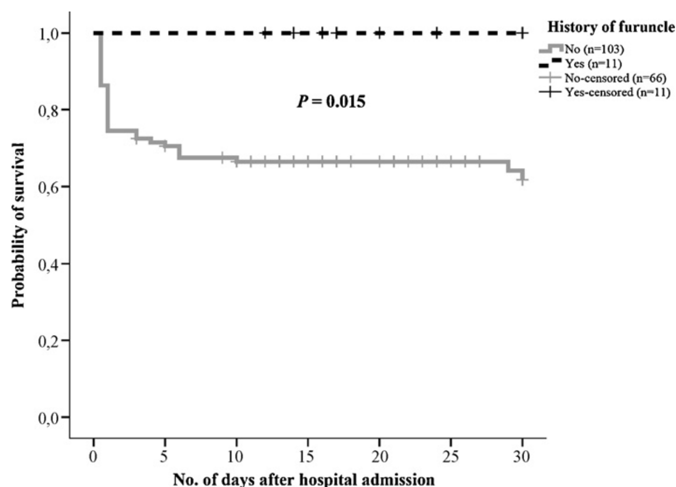

Fig. 1. Probability of survival among patients with Panton-Valentine leukocidin-associated necrotizing pneumonia, based on the presence of a history of furuncle. Patients who survived were censored at discharge from the hospital. The difference in survival probability was tested for statistical significance using Fisher's exact test. The Mantel-Cox method could not be used because all the cases with a history of furuncle were censored.

hospital admission (p = 0.015) (Fig. 1). The need for inotrope support or mechanical ventilation was significantly less frequent in patients with a history of furuncle than in those without (Table 1).

The findings demonstrate that a history of furuncle, considered here as a surrogate marker for the presence of pre-existing immunity to PVL in the patients, is associated with a much better outcome in PVL-associated necrotizing pneumonia. This observation is consistent with the experimental models, in which PVL vaccine has been found to protect mice against PVL-associated lung infection [8]. Moreover, on examining a series of 50 patients with PVL-associated necrotizing pneumonia, Gillet et al. reported that furuncle history was more frequent, although not significant, in patients who survived than in those who died [9]. On the other hand, the inefficacy of pre-existing immunity to PVL to prevent SSTI recurrence, which was recently reported by Hermos et al., has led them to conclude that the "inclusion of the LukS component of PVL in experimental vaccines under development for *S. aureus* infection may provide little benefit" [5]. However, their study did not address the question of the influence of immunity to PVL on disease outcome.

4. Conclusions

Our results strongly suggest a protective role of PVL-directed immunity in severe systemic PVL-associated disease. This finding brings new arguments in the controversy about PVL-directed vaccine strategy. Although recently published data suggest that the use of PVL as a vaccine target in humans should not be aimed at preventing SSTI [5], anti-PVL vaccine could still provide strong clinical benefit in invasive, life-threatening infections, such as necrotizing pneumonia, and should be encouraged.

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Contributors: JPR, FL, GL, and JE contributed in writing the article; JE and NS contributed in study design; NS, GL, FV, and JE contributed in data acquisition; JPR, NS, and FL contributed in data analysis and interpretation.

Conflict of interest statement: We declare that we have no conflict of interest.

References

[1] Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 2007;298(15):1763–71.
[2] Tristan A, Bes M, Meugnier H, Lina G, Bozdogan B, Courvalin P, et al. Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. Emerg Infect Dis 2007;13(4):594–600.
[3] Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu Rev Microbiol 2010;64:143–62.
[4] ClinicalTrials.gov. *Staphylococcus aureus* toxoids phase 1–2 vaccine trial. http://www.clinicaltrials.gov/ct2/show/NCT01011335?term=NABI&rank=5 [accessed 01.01.11].
[5] Hermos CR, Yoong P, Pier GB. High levels of antibody to Panton-Valentine leukocidin are not associated with resistance to *Staphylococcus aureus*-associated skin and soft-tissue infection. Clin Infect Dis 2010;51(10):1138–46.
[6] Yoong P, Pier GB. Antibody-mediated enhancement of community-acquired methicillin-resistant *Staphylococcus aureus* infection. Proc Natl Acad Sci USA 2010;107(5):2241–6.
[7] Masiuk H, Kopron K, Grumann D, Goerke C, Kolata J, Jursa-Kulesza J, et al. Association of recurrent furunculosis with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus*. J Clin Microbiol 2010;48(5):1527–35.
[8] Brown EL, Dumitrescu O, Thomas D, Badiou C, Koers EM, Choudhury P, et al. The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by *Staphylococcus aureus* USA300. Clin Microbiol Infect 2009;15(2):156–64.
[9] Gillet Y, Vanhems P, Lina G, Bes M, Vandenesch F, Floret D, et al. Factors predicting mortality in necrotizing community-acquired pneumonia caused by *Staphylococcus aureus* containing Panton-Valentine leukocidin. Clin Infect Dis 2007;45(3):315–21.

ANNEXE IV

T-cell response to superantigen restimulation during menstrual toxic shock syndrome

Rasigade JP, Thomas D, Perpoint T, Peyramond D, Chidiac C, Etienne J, Vandenesch F, Lina G, Ferry T

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SHORT COMMUNICATION

T-cell response to superantigen restimulation during menstrual toxic shock syndrome

Jean-Philippe Rasigade[1], Damien Thomas[1], Thomas Perpoint[2], Dominique Peyramond[2], Christian Chidiac[2], Jerome Etienne[1], François Vandenesch[1], Gerard Lina[1] & Tristan Ferry[1,2]

[1]INSERM U851, National Reference Center for Staphylococci, University of Lyon Claude Bernard, Lyon, France; and [2]Department of Infectious and Tropical Diseases, Croix-Rousse Hospital, Hospices Civils de Lyon, Lyon, France

Correspondence: Jean-Philippe Rasigade, INSERM U851/Centre National de Référence des Staphylocoques, Faculté de Médecine Lyon-Est, 7 rue Guillaume Paradin, 69372 Lyon Cedex 8, France. Tel.: +33 478 77 86 57; fax: +33 478 77 86 58; e-mail: jean-philippe.rasigade@chu-lyon.fr

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Staphylococcus aureus; nonmenstrual TSS; TSST-1; staphylococcal enterotoxin B; Vβ signature.

Abstract

Menstrual toxic shock syndrome (MTSS) is a severe toxin-mediated disease associated with *Staphylococcus aureus* producing toxic shock syndrome toxin 1 (TSST-1), a superantigen that mediates a potent activation of Vβ-2 T cells. In animal models, superantigen treatment of responsive T cells induces their initial proliferation, followed by unresponsiveness upon further superantigen stimulation. To determine whether T cell unresponsiveness occurs in humans during the acute phase of MTSS, we collected T cells from a patient with MTSS and restimulated them *ex vivo* with recombinant TSST-1. The expansion of T cells collected during the acute phase of disease was compared with positive controls including basal-state T cells (collected 70 days after MTSS) restimulated with TSST-1, and T cells stimulated with enterotoxin B superantigen. We found that TSST-1-induced expansion of acute phase T cells was not inferior to that observed in positive controls. We conclude that T cells were still reactive to TSST-1 during the acute phase of MTSS in this patient. As the persistence of TSST-1 production could thus be associated with further expansion of TSST-1-reactive T cells and a rapid worsening of symptoms, this study adds further support to the need for immediate eradication of the focus of infection as soon as MTSS is suspected.

Staphylococcal toxic shock syndrome (TSS) is a severe, toxin-mediated systemic disease caused by superantigenic toxins secreted by *Staphylococcus aureus*. Clinical features include the sudden onset of fever, cutaneous signs (rash followed by desquamation), hypotension and multi-system involvement (McCormick *et al*., 2001). Menstrual toxic shock syndrome (MTSS) involves young, healthy menstruating women, especially those using tampons. MTSS is associated with noninvasive vaginal infection by a *S. aureus* strain producing the toxic shock syndrome toxin 1 (TSST-1), a superantigen that has the ability to cross mucosa. Although this disease can cause significant morbidity, fatal outcome is rare (Descloux *et al*., 2008). On the other hand, TSSs that occur without relation to menstruation, designated as nonmenstrual (NM-) TSSs, carry a fatality rate of over 20% (Descloux *et al*., 2008). NMTSS is mostly associated with TSST-1, but also with staphylococcal enterotoxins such as SEB. Postoperative NMTSS can arise from *S. aureus* infection of the surgical wound, often with little, if any, local signs of inflammation (Bartlett *et al*., 1982).

Superantigens mediate a potent activation of immune cells by cross-linking major histocompatibility complex class II molecules on antigen-presenting cells to the Vβ domain of the T-cell receptor (McCormick *et al*., 2001). These interactions activate both T cells and antigen-presenting cells at orders of magnitude above antigen-specific activation, followed by uncontrolled cytokine release, which is responsible for the clinical symptoms (Miethke *et al*., 1993). Superantigens activate specific Vβ subsets of T cells (e.g. TSST-1 targets Vβ-2 T cells, whereas SEB targets Vβ-3, Vβ-14 and Vβ-17 T cells), thus allowing the determination of Vβ signatures that help to show which superantigen is responsible for a given TSS (Ferry *et al*., 2008b). T-cell response to superantigen stimulation has been studied extensively in animal models, in which superantigen treatment induces initial proliferation of responsive Vβ T cells followed by a state of unresponsiveness (Renno *et al*., 1995; Waclavicek

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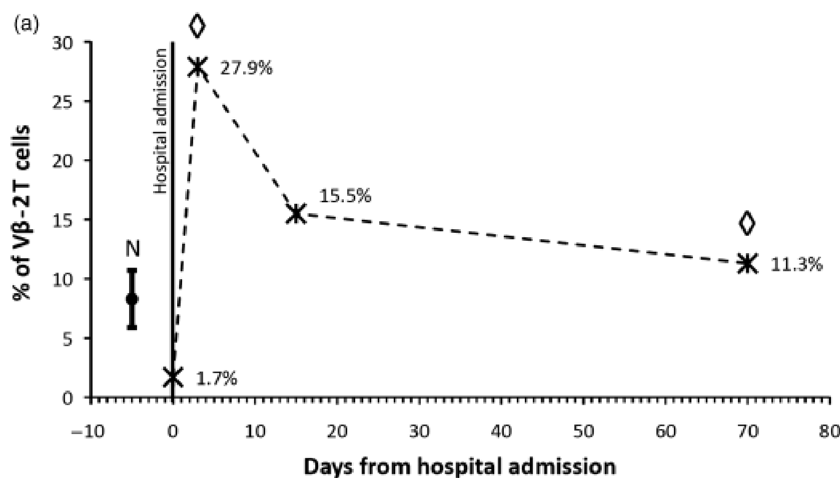
et al., 2009). However, we lack data on whether this unresponsiveness occurs *in vivo* during human TSS. In the present study, we investigated the response, upon *ex vivo* restimulation with TSST-1, of Vβ-2 T cells collected from a patient with MTSS, using flow cytometry quantification of Vβ-2 T-cell expansion.

An 18-year-old woman was admitted via the emergency care unit on 5 July 2007 with a 2-day history of pruritic rash of the extremities, sore throat, fever and nausea without vomiting. On admission, the temperature was 39 °C, the blood pressure was 86/48 mmHg and the pulse rate was 161 beats min^{-1}. The throat and tongue were red, but not the tonsils. No other clinical abnormalities were noted. After immediate fluid resuscitation, the blood pressure rose to 104/60 mmHg and the pulse rate fell to 110 beats min^{-1}. MTSS was suspected because she was menstruating and wearing a tampon. The tampon was immediately removed and she was given cloxacillin and linezolid. A vaginal swab grew methicillin-susceptible *tst*-positive *S. aureus*. Blood cultures remained sterile. The erythroderma faded, leaving scarlatiniform lesions on the arms and legs that desquamated 7 days after hospital admission. All diagnostic criteria for definitive staphylococcal TSS were thus fulfilled. She recovered without any sequelae.

Blood samples were collected on admission and on days 3, 15 and 70. The proportions of Vβ-2 T cells were determined as described elsewhere (MacIsaac *et al.*, 2003). The responsiveness of the patient's T cells upon restimulation with superantigens was investigated in blood samples collected on days 3 (acute phase) and 70 (convalescent phase). Convalescent phase T cells were used as positive control, assuming that Vβ-2 T cells had returned to basal state and were thus fully reactive to TSST-1 stimulation. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient sedimentation. Before restimulation, a subset of cells were stained by R-phycoerythrincyanin 5-labelled antibody to T-cell marker CD3, and phycoerythrin-labelled antibodies to Vβ-2 and Vβ-14 (Immunotech/



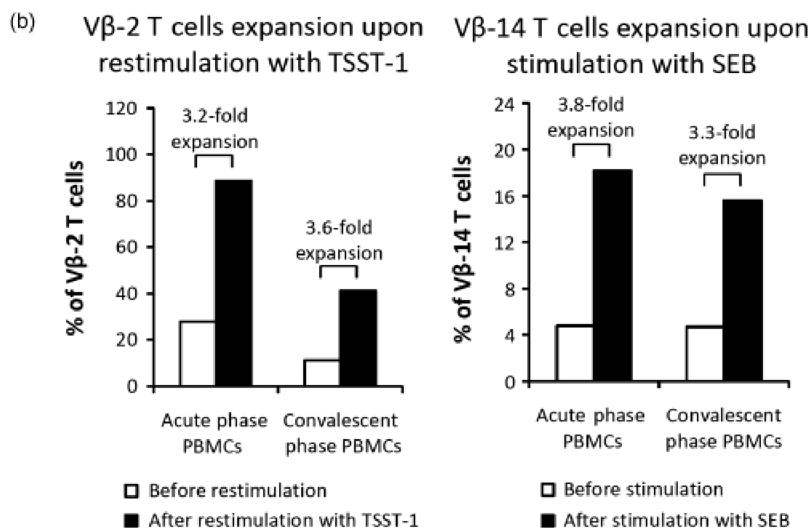


Fig. 1. (a) Kinetics of the percentage of TSST-1 reactive, Vβ-2 T cells in a patient with MTSS. *N*, normal mean percentage ± 2 SD of Vβ-2 T cells. ◇, *ex vivo* restimulation of PBMCs with TSST-1. (b) Expansion of TSST-1 reactive, Vβ-2 T cells upon *ex vivo* restimulation with TSST-1, and of SEB reactive, Vβ-14 T cells upon *ex vivo* stimulation with SEB (comparator). PBMCs sampled on day 3 (acute phase) and day 70 (convalescent phase) after hospital admission were treated *ex vivo* with 100 ng mL^{-1} TSST-1 or 100 ng mL^{-1} SEB for 48 h, and further cultivated in superantigen-free medium for 9 days to allow for cell expansion and re-expression of T-cell receptors. Cells were labelled using antibodies against the T-cell marker CD3 and the Vβ-2 and Vβ-14 subdomains of the T-cell receptor, and analysed by flow cytometry. Results are expressed as the percentage of Vβ-2 and Vβ-14 T cells among CD3-positive cells. The expansion upon restimulation with TSST-1 of Vβ-2 T cells collected during the acute phase of disease was similar to that observed in comparator conditions, demonstrating that these cells were fully responsive to TSST-1 challenge.

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Beckman Coulter, Fullerton, CA). The percentages of TSST-1-reactive Vβ-2 T cells and SEB-reactive Vβ-14 T cells were determined by flow cytometry using a FACScan cytometer and CELLQUEST v3.0 software (Becton Dickinson Biosciences, San Jose, CA). The remaining PBMCs ($2 \times 10^6 \, mL^{-1}$) were incubated in RPMI-1640 medium (Invitrogen, Paisley, UK) containing $100 \, ng \, mL^{-1}$ TSST-1 or SEB. TSST-1 and SEB were produced in our laboratory as recombinant proteins, as described elsewhere (Thomas *et al.*, 2009). SEB was used as comparator because it targets T-cell populations, including Vβ-14 that had not been previously stimulated *in vivo*. After 48 h, cells were washed twice and further cultured for 9 days in superantigen-free medium to allow for cell expansion and re-expression of internalized T-cell receptors (Kappler *et al.*, 1989). The proportions of Vβ-2 and Vβ-14 T cells were then determined as described above, and the expansion of acute phase Vβ-2 T cells after TSST-1 restimulation was compared with that of positive control cells, namely TSST-1-restimulated, convalescent phase Vβ-2 T cells, and SEB-stimulated Vβ-14 T cells.

On admission, Vβ-2 T cells were 1.7% (normal $8.3 \pm 2.4\%$) (Ferry *et al.*, 2008b); on day 3, 27.9%; on day 15, 15.5%; on day 70, 11.3% (Fig. 1a). The time course of Vβ-2 T cells percentage was consistent with that described in other reports of MTSS, with an initial depletion of Vβ-2 T cells, followed by their expansion and progressive decrease (Ferry *et al.*, 2008a).

After restimulation with TSST-1 of acute phase PBMCs, the percentage of T cells expressing Vβ-2 increased from 27.9% to 88.5% (3.2-fold increase) (Fig. 1b); in convalescent phase PBMCs, this percentage increased from 11.3% to 41.1% (3.6-fold increase). After SEB treatment, Vβ-14 T cells increased from 4.8% to 18.2% (3.8-fold increase) in acute phase PBMCs, and from 4.7% to 15.6% (3.3-fold increase) in convalescent phase PBMCs.

TSST-1-reactive T-cell expansion upon TSST-1 restimulation was therefore equally significant in PBMCs collected during the acute phase and the convalescent phase of human MTSS, and comparable to SEB-induced expansion of SEB-reactive, previously unstimulated T cells. This observation is in contrast with the conclusions of a previous report stating that human TSST-1-reactive T cells are anergic upon TSST-1 restimulation (Mahlknecht *et al.*, 1996). A possible reason for this discrepancy is that the authors did not use Vβ-2 T-cell expansion as the primary readout, but the amount of activated, blast T-cell formation within the Vβ-2 T-cell population.

Our findings suggest that in some patients, Vβ-2 T cells are still responsive to TSST-1 stimulation during the acute phase of TSS. As the persistence of TSST-1 production in such patients could thus be associated with further expansion of TSST-1-reactive T cells and a rapid worsening of symptoms, this study adds support to the need for antitoxin therapy and the immediate eradication of the focus of infection as soon as TSS is suspected. While focus eradication in MTSS can be easily achieved through tampon removal, more invasive procedures may be warranted in postoperative NMTSS, including reopening and debridement of the surgical wound, even in the absence of local signs of infection.

Acknowledgements

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References

Bartlett P, Reingold AL, Graham DR, Dan BB, Selinger DS, Tank GW & Wichterman KA (1982) Toxic shock syndrome associated with surgical wound infections. *J Am Med Assoc* **247**: 1448–1450.

Descloux E, Perpoint T, Ferry T, Lina G, Bes M, Vandenesch F, Mohammedi I & Etienne J (2008) One in five mortality in non-menstrual toxic shock syndrome vs. no mortality in menstrual cases in a balanced French series of 55 cases. *Eur J Clin Microbiol* **27**: 37–43.

Ferry T, Thomas D, Bouchut JC, Lina G, Vasselon-Raina M, Dauwalder O, Gillet Y, Vandenesch F, Floret D & Etienne J (2008a) Early diagnosis of staphylococcal toxic shock syndrome by detection of the TSST-1 Vbeta signature in peripheral blood of a 12-year-old boy. *Pediatr Infect Dis J* **27**: 274–277.

Ferry T, Thomas D, Perpoint T, Lina G, Monneret G, Mohammedi I, Chidiac C, Peyramond D, Vandenesch F & Etienne J (2008b) Analysis of superantigenic toxin Vbeta T-cell signatures produced during cases of staphylococcal toxic shock syndrome and septic shock. *Clin Microbiol Infec* **14**: 546–554.

Kappler J, Kotzin B, Herron L, Gelfand EW, Bigler RD, Boylston A, Carrel S, Posnett DN, Choi Y & Marrack P (1989) Vbeta-specific stimulation of human T cells by staphylococcal toxins. *Science* **244**: 811–813.

MacIsaac C, Curtis N, Cade J & Visvanathan K (2003) Rapid analysis of the Vbeta repertoire of CD4 and CD8T lymphocytes in whole blood. *J Immunol Methods* **283**: 9–15.

Mahlknecht U, Herter M, Hoffmann MK, Niethammer D & Dannecker GE (1996) The toxic shock syndrome toxin-1 induces anergy in human T cells *in vivo*. *Hum Immunol* **45**: 42–45.

McCormick JK, Yarwood JM & Schlievert PM (2001) Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol* **55**: 77–104.

Miethke T, Wahl C, Regele D, Gaus H, Heeg K & Wagner H (1993) Superantigen mediated shock: a cytokine release syndrome. *Immunobiology* **189**: 270–284.

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Published by Blackwell Publishing Ltd. All rights reserved

Renno T, Hahne M & MacDonald HR (1995) Proliferation is a prerequisite for bacterial superantigen-induced T cell apoptosis *in vivo*. *J Exp Med* **181**: 2283–2287.

Thomas D, Dauwalder O, Brun V, Badiou C, Ferry T, Etienne J, Vandenesch F & Lina G (2009) *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. *Infect Immun* **77**: 2043–2050.

Waclavicek M, Stich N, Rappan I, Bergmeister H & Eibl MM (2009) Analysis of the early response to TSST-1 reveals Vbeta-unrestricted extravasation, compartmentalization of the response, and unresponsiveness but not anergy to TSST-1. *J Leukocyte Biol* **85**: 44–54.

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ANNEXE V

DNA microarray-based characterization of Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* from Italy

Sanchini A, Campanile F, Monaco M, Cafiso V, Rasigade JP, Laurent F, Etienne J, Stefani S, Pantosti A

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ARTICLE

DNA microarray-based characterisation of Panton–Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* from Italy

A. Sanchini · F. Campanile · M. Monaco · V. Cafiso · J.-P. Rasigade · F. Laurent · J. Etienne · S. Stefani · A. Pantosti

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Abstract Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates are widespread in many countries, with varying distribution and epidemiology. The aim of this study was to collect and characterise the CA-MRSA isolates circulating in Italy, since only some case reports have been published. Eighteen Panton–Valentine-positive CA-MRSA isolates were collected from different Italian hospitals during the period 2005–2009 from severe infections (skin and soft tissue infections, n=10; necrotising pneumonia, n=7; and sepsis, n=1). Accessory gene regulator (*agr*) typing, staphylococcal cassette chromosome (SCC) *mec* typing, *spa* typing, multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and DNA microarray were applied to categorise isolates into clones and to compare the relevant genetic features of each clone. Six different clones were identified, the most common (7 out of 18 isolates, 38.8%) being *agr*I/ST8/SCC*mec*IV, corresponding to the USA300 clone. Six out of the seven USA300 isolates did not harbour the arginine catabolic mobile element (ACME). Four strains (22.2%) were *agr*III/ST80/SCC*mec*IV, corresponding to the European clone. Two of the other clones, namely, *agr*III/ST88/SCC*mec*V and *agr*III/ST772/SCC*mec*V, corresponded to CA-MRSA clones rarely found in other countries and probably originating from Africa or the Indian subcontinent. The results of microarray hybridisations showed that the distribution of resistance genes and other virulence factors was specific to each clone. Some characteristics could be exploited as specific markers for a clone or a group of isolates, e.g. the *mer* operon, recovered only in ACME-negative USA300 strains. DNA microarray contributed to a more complete description of the variety of different CA-MRSA clones circulating in Italy.

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A. Sanchini · M. Monaco · A. Pantosti (✉)
Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità,
299, Viale Regina Elena,
00161 Rome, Italy
e-mail: annalisa.pantosti@iss.it

F. Campanile · V. Cafiso · S. Stefani
Department of Biomedical Science, Section of Microbiology, University of Catania,
83, Via Androne,
95124 Catania, Italy

J.-P. Rasigade · F. Laurent · J. Etienne
Inserm U851, Faculté de Médecine Lyon Est, University of Lyon,
7, Rue Guillaume Paradin,
69008 Lyon, France

J.-P. Rasigade · F. Laurent · J. Etienne
French National Reference Center for Staphylococci,
Hospices Civils de Lyon,
59, Boulevard Pinel,
69003 Lyon, France

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) appeared and spread rapidly after the introduction of methicillin in clinical use in 1960, becoming one of the most prevalent pathogens in healthcare settings [1]. MRSA causes serious infections such as sepsis and pneumonia in hospitalised patients, with specific risk factors [2]. The emergence of MRSA causing community-acquired infec-

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tions (CA-MRSA), particularly in healthy individuals without risk factors, has been reported since the end of the 1990s in the United States and in other countries [3]. Typical clinical presentations of CA-MRSA are skin and soft-tissue infections (SSTIs), including furuncles and skin abscesses, or deep-seated infections such as necrotising pneumonia, bone infections, sepsis and meningitis [2]. CA-MRSA strains appear phenotypically and genotypically different from hospital-acquired MRSA (HA-MRSA), although these differences have blurred over the recent years [2, 3]. CA-MRSA strains are generally susceptible to antibiotics other than beta-lactams and harbour staphylococcal cassette chromosome (SCC) *mec* type IV, V or VII [4]. On the other hand, HA-MRSA are generally multidrug-resistant and contain SCC*mec* type I, II or III, although in the last few years, some HA-MRSA clones containing SCC*mec* type IV have spread in Europe, e.g. EMRSA15 and the Lyon clone (sequence type [ST] 8) [5, 6]. Besides, antibiotic resistance within CA-MRSA is increasing [7]. Characteristically, CA-MRSA strains carry the genes encoding the Panton–Valentine leukocidin (PVL), a secreted virulence factor which causes polymorphonuclear leukocytes lysis and tissue necrosis [8].

Several different clones of CA-MRSA are spread worldwide. In the United States, the USA300 clone (ST8) is responsible for the major part of community-acquired SSTIs and for outbreaks in the community and in hospitals [3]. The presence of type I arginine catabolic mobile element (ACME) has been proposed to contribute to the fitness and transmissibility of the USA300 clone [9].

In Europe, CA-MRSA infections are less frequent than in the United States and are mainly associated with the European clone ST80 [10]. In Italy, some case reports or small studies have been published [11–15], but a more complete overview of CA-MRSA is lacking.

The aim of this study was: (i) to collect CA-MRSA strains from serious infections and characterise them by phenotypic and genotypic methods; (ii) to compare the results with those obtained applying a DNA microarray technique which permits to simultaneously identify a wide set of virulence factors, antibiotic resistance determinants and typing markers; and (iii) to recognise common or unique features among CA-MRSA isolates.

Materials and methods

Bacterial isolates, antimicrobial susceptibility and biofilm production testing

Eighteen CA-MRSA isolates were collected from April 2005 to October 2009. The isolates were referred by different Italian hospital laboratories all over the country on the basis of the type and the severity of the infections that appeared typical of CA-MRSA.

The isolates were tested for antimicrobial susceptibility by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [16]. When EUCAST breakpoints were not available for specific antibiotics, Clinical and Laboratory Standards Institute (CLSI) [17] or British Society for Antimicrobial Chemotherapy (BSAC) [18] breakpoints were applied. The agents tested included: cefoxitin, gentamicin, ciprofloxacin, erythromycin, kanamycin, clindamycin, tetracycline, rifampicin, trimethoprim–sulfamethoxazole, fusidic acid, fosfomycin and mupirocin. Minimum inhibitory concentrations of vancomycin, teicoplanin, linezolid, quinupristin–dalfopristin and daptomycin were performed using the E-test method (bioMérieux, Marcy-l’Etoile, France).

Biofilm production was determined spectrophotometrically as described elsewhere [15]. The isolates were categorised according to the optical density (OD) reading. OD values $\leq$0.12 corresponded to biofilm non-producers, OD=0.13–0.2 to weak producers, OD=0.2–0.4 to medium producers and OD>0.4 to strong producers [15].

Characterisation of the isolates by PCR

S. aureus genomic DNA was extracted with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). *S. aureus* species and methicillin resistance were confirmed by polymerase chain reaction (PCR) assays detecting *nuc* and *mecA* genes [19]. PCR assays were performed to detect genes encoding PVL, *agr* alleles, ACME-specific *arcA* gene and genes encoding capsular types 5 and 8 [15, 19, 20]. Adhesin and toxin genes content was also evaluated as previously described [15]. SCC*mec* types were determined by combining the detection of *ccr* genes and *mec* complex genes as described elsewhere [19].

Molecular typing of the isolates

S. aureus protein A (*spa*) gene typing and multi-locus sequence typing (MLST) were performed on the isolates as described elsewhere [19]. Pulsed-field gel electrophoresis (PFGE) was applied to the seven CA-MRSA isolates belonging to ST8 to distinguish USA300 isolates [21]. USA300 strain FPR3757 was used as the control [20]. PFGE patterns were analysed following established criteria [21, 22].

DNA microarray hybridisation

DNA microarray hybridisation was performed at the French National Reference Center for Staphylococci (Lyon, France), on the ArrayTube platform (Identibac *S. aureus*

Genotyping, Alere, Sevres, France) [23, 24]. The array contains covalently immobilised probes specific for approximately 180 genes and 300 alleles of *S. aureus*, including typing targets, resistance genes, toxins and microbial surface components [23, 24]. The genes considered in this study and their function are listed in Supplementary Table S1.

Results and discussion

Out of 18 isolates, 8 (44.5%) were from males and 10 (55.5%) were from females (Table 1). The age of the patients ranged from 3 to 66 years (median 29.5 years). All patients lived in the community and reported no recent hospitalisation. The geographical distribution of the cases on the map of Italy is shown in Fig. 1. There was no apparent epidemiological relationship between cases, although in two areas, several cases were reported by the hospital laboratories, probably due to a better awareness of the problem.

All isolates were confirmed as PVL-positive MRSA by molecular methods. Only one strain (Sau65) harboured the ACME-specific *arcA* gene.

All isolates were resistant to at least one non-beta-lactam antibiotic among those tested: 12 isolates were resistant to fosfomycin (66.6%), ten to kanamycin (55.5%), six to tetracycline (33.3%), five to gentamicin (27.7%), four to erythromycin (22.2%), four to fusidic acid (22.2%), two to ciprofloxacin (11.1%), one to mupirocin (5.5%) and one to clindamycin (5.5%) (Table 1). All isolates were susceptible to rifampicin, trimethoprim–sulfamethoxazole, vancomycin, teicoplanin, quinupristin–dalfopristin, linezolid and daptomycin.

Table 1 Clinical information and phenotypic and genotypic characteristics of the 18 community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains

Isolate	Sex	Age	Isolation year	Type of infection	*agr* allele	ST	*spa* type	SCC*mec* type	Resistance to non-beta-lactam antibiotics[a]	Capsular type	Biofilm[b]
Sau17	M	16	2006	Necrotising pneumonia, sepsis and meningitis	I	8	t008	IV	fos	5	MP
Sau18	F	21	2007	Necrotising pneumonia	I	8	t008	IV	fos	5	NP
Sau14	M	16	2005	Necrotising pneumonia	I	8	t008	IV	fos	5	WP
Sau16	M	3	2006	SSTI	I	8	t008	IV	fos	5	MP
Sau32	M	11	2008	SSTI	I	8	t008	IV	fos	5	SP
Sau25	F	30	2008	Sepsis	I	8	t008	IV	fos	5	SP
Sau65	F	28	2009	SSTI	I	8	t008	IV	ery, kan, mup, fos	5	ND
Sau19	F	66	2007	Necrotising pneumonia	III	80	t044	IV	kan, tet, fus	8	MP
Sau21	F	50	2008	Necrotising pneumonia	III	80	t2453	IV	kan, tet, fus	8	MP
Sau58	M	41	2009	SSTI	III	80	t044	IV	ery, cli, kan, tet, fus	8	ND
Sau57	M	65	2009	SSTI	III	80	t044	IV	kan, tet, fus	8	ND
Sau24	M	25	2008	Necrotising pneumonia	III	88	t2526	V	kan, gen	8	SP
Sau27	F	44	2008	SSTI	III	88	t002	V	kan, gen	8	SP
Sau30	F	54	2008	SSTI	II	772	t345	V	ery, kan, gen, cip, fos	5	SP
Sau33	M	58	2008	SSTI	II	772	t345	V	ery, kan, gen, cip, fos	5	SP
Sau22	F	29	2008	SSTI	II	5	t319	IV	tet, fos	5	MP
Sau31	F	3	2008	SSTI	II	5	t002	V	kan, gen, tet, fos	5	MP
Sau15	F	37	2008	Necrotising pneumonia	III	30	t755	IV	fos	8	NP

[a] EUCAST breakpoints were applied [16], except for kanamycin and mupirocin, for which CLSI [17] and BSAC [18] breakpoints were applied, respectively. fos, fosfomycin; ery: erythromycin; kan: kanamycin; mup: mupirocin; tet: tetracycline; fus: fusidic acid; cli: clindamycin; gen: gentamicin; cip: ciprofloxacin

[b] NP, non-producer; WP, weak producer; MP, medium producer; SP, strong producer (see text); ND, not determined; SSTI, skin and soft tissue infection; ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*

Fig. 1 Geographical distribution of the community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) cases. The numbers within each placemarker represent the number of cases

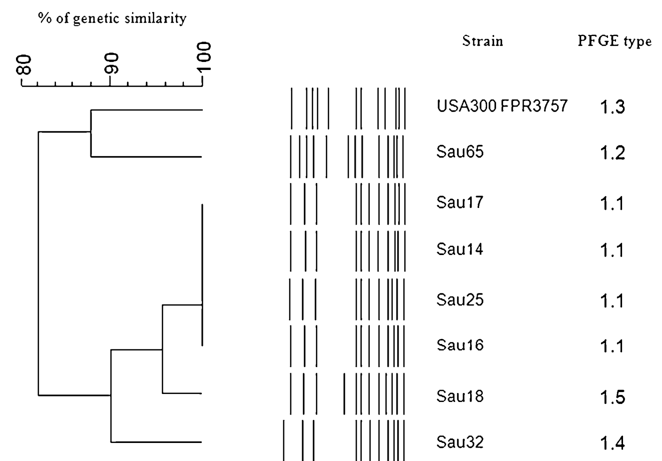

Fig. 2 Phylogenetic analysis of pulsed-field gel electrophoresis (PFGE) profiles obtained with the *agr*I/ST8/SCC*mec*IV isolates. The dendrogram was constructed with PFGE profiles by similarity and clustering analysis by the unweighted pair group method with arithmetic mean (UPGMA) and the Dice coefficient, by using a band tolerance of 1.5%. The percentage of genetic similarity is shown above the dendrogram. Isolates showed a percentage of similarity >80%

Molecular typing

Thirteen isolates harboured SCC*mec* type IV and five harboured SCC*mec* type V. *agr* alleles I, II and III were found. The isolates belonged to six different STs: seven isolates belonged to *agr*I/ST8/SCC*mec*IV, four isolates to *agr*III/ST80/SCC*mec*IV, two isolates each belonged to *agr*III/ST88/SCC*mec*V, *agr*II/ST772/SCC*mec*V and *agr*II/ST5/SCC*mec*IV–V, and one isolate to *agr*III/ST30/SCC*mec*IV. Isolates of a given ST shared the same capsular type, *agr* allele, *spa* type (or correlated) and SCC*mec* type, except for ST5 isolates, which harboured two different SCC*mec* types (Table 1).

PFGE analyses performed on the ST8 isolates revealed that all of the isolates were related, since they had less than four bands different. Five subtypes (from 1.1 to 1.5) were recovered, which showed a percentage of similarity >80% (exactly, 82.38%) (Fig. 2).

Microarray analyses

The microarray results are synthesised in Figures 3, 4 and 5. With respect to antibiotic resistance, a strong correlation was found between phenotypical resistance and the presence of corresponding resistance genes by microarray. With respect to biofilm, although all isolates possessed the *ica* operon involved in biofilm formation, phenotypical tests showed different rates of biofilm production, likely reflecting differences in gene expression or in other characteristics among the isolates. The molecular characterisation obtained by PCR (*mecA*, PVL, *arcA*, capsular, ACME, *agr* SCC*mec* genes, other toxin and adhesion genes) was concordant with the microarray results, confirming the high fidelity of this latter approach.

Despite the genetic diversity of the isolates, some genes were homogeneously distributed in all strains, including genes encoding leukocidins (*lukS-F*, *hlgA*, *lukX*, *lukY*), haemolysins (*hla*, *hld*), proteases (*aur*, *sspA-B-P*), adhesion proteins (*clfA-B*, *ebh*, *eno*, *fib*, *fnbA-B*, *sdrC*, *vwb* and *sasG*) and immune-evasion factors (*mprF* and *isdA*). These genes have an almost ubiquitary distribution in *S. aureus*, as shown by Monecke et al. by the microarray hybridisation of 100 clinical strains, including methicillin-susceptible *S. aureus* (MSSA), CA-MRSA and HA-MRSA belonging to different genetic lineages [23].

In our collection, only two strains possessed an intact beta-haemolysin gene (*hlb*), while 16 others harboured *hlb* genes truncated after the insertion of phage-borne genes, such as *entA* (enterotoxin A), or immune-evasion genes, such as *sak* (staphylokinase), *chp* (chemotaxis inhibitory protein) or *scn* (staphylococcal complement inhibitor).

The overall presence of antibiotic resistance determinants and enterotoxin genes or clusters was different between, and sometimes within, the clones, in line with their location on mobile genetic elements.

The microarray results are analysed below according to the six different *agr*/ST/SCC*mec* combinations found:

1. *agr*I/ST8/SCC*mec*IV

   Isolates with these characteristics were the most prevalent in our collection. PFGE was performed in order to evaluate if the seven ST8 CA-MRSA isolates belonged or were related to the USA300 clone, since

Isolate	Clonal Group	*blaZ-I-R*	*ermA*	*ermB*	*ermC*	*linA*	*msrA*	*mefA*	*mpbBM*	*vatA-B*	*vga*	*vgb*	*aacA-aphD*	*aadD*	*aphA-3*	*sat*	*dfrA*	*far1*	*mupR*	*tetK*	*tetM*	*cat*	*fexA*	*cfr*	*fosB*	*qacA*	*qacC*	*vanA-B-Z*	*mer* operon	ACME
		Antibiotic resistance genes																												
Sau17	*agr*I ST8 IV	•																							•				•	
Sau18	*agr*I ST8 IV	•																							•				•	
Sau14	*agr*I ST8 IV	•																							•				•	
Sau16	*agr*I ST8 IV	•																							•				•	
Sau32	*agr*I ST8 IV	•																							•				•	
Sau25	*agr*I ST8 IV	•																		•					•				•	
Sau65	*agr*I ST8 IV	•					•		•						•	•			•						•					•
Sau19	*agr*III ST80 IV	•													•	•		•		•										
Sau21	*agr*III ST80 IV	•													•	•		•		•										
Sau58	*agr*III ST80 IV	•			•										•	•		•		•										
Sau57	*agr*III ST80 IV	•													•	•		•		•										
Sau24	*agr*III ST88 V	•											•																	
Sau27	*agr*III ST88 V	•											•																	
Sau30	*agr*II ST772 V	•					•		•				•		•	•									•					
Sau33	*agr*II ST772 V	•					•		•				•		•	•									•					
Sau22	*agr*II ST5 IV																•				•				•		•			
Sau31	*agr*II ST5 V												•							•					•					
Sau15	*agr*III ST30 IV	•																							•					

Fig. 3 Presence of antibiotic-resistance genes and arginine catabolic mobile element (ACME) in the CA-MRSA strains. The dots indicate positive results for probe hybridization. The function of each gene is listed in Supplementary Table S1

Isolate	Clonal Group	*tsst-1*	*entA*	*entP*	*entB*	*entC-L*	*entD-J-R*	*entE*	*egc*	*entH*	*entK-Q*	*lukS-F*	*hlgA*	*lukF-PV P83*	*lukM*	*lukD*	*lukE*	*lukX*	*lukY* var 1	*lukY* var 2	*hla*	*hld*	*hlb intact*	*hlb truncated*	*sak*	*chp*	*scn*	*etA*	*etB*	*etD*	*edinA*	*edinB*	*edinC*
		Superantigenic toxins										Leukocidins									Haemolysins				Phagic genes			Exfoliative toxins			*edin* factors		
Sau17	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•	•	•									
Sau18	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•	•	•									
Sau14	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau16	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau32	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau25	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau65	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau19	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau21	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau58	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau57	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau24	*agr*III ST88 V			•								•	•			•	•	•	•		•	•		•	•	•	•						
Sau27	*agr*III ST88 V			•								•	•			•	•	•	•		•	•		•	•	•	•						
Sau30	*agr*II ST772 V		•			•			•			•	•					•	•		•	•					•						
Sau33	*agr*II ST772 V		•			•			•			•	•					•	•		•	•					•						
Sau22	*agr*II ST5 IV								•			•	•			•	•	•	•		•	•		•	•	•	•						
Sau31	*agr*II ST5 V			•			•		•			•	•			•	•	•	•		•	•		•	•		•						
Sau15	*agr*III ST30 IV								•			•	•					•		•	•	•		x	•	•	•						

Fig. 4 Presence of genes encoding miscellaneous virulence factors in the CA-MRSA strains. The dots indicate positive results for probe hybridization and the X indicates an ambiguous result. The function of each gene is listed in Supplementary Table S1

Isolate	Clonal Group	Capsule 5	Capsule 8	*aur*	*splA*	*splB*	*splE*	*sspA-B*	*sspP*	*icaA*	*icaC*	*icaD*	*bap*	*bbp*	*clfA*	*clfB*	*cna*	*ebh*	*ebpS*	*eno*	*fib*	*fib* MRSA25	*fnbA*	*fnbB*	*map*	*sdrC*	*vwb*	*sasG*	*mprF*	*isdA*	*isdA* 252	*hsdS1*	*hsdS2*	*hsdS3*
				Proteases						Biofilm				MSCRAMMs															Immune-evasion factors					
Sau17	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau18	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau14	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau16	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau32	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau25	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau65	*agr*I ST8 IV	•		x	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau19	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	
Sau21	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	
Sau58	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			x	
Sau57	*agr*III ST80 IV		•	x	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•				
Sau24	*agr*III ST88 V		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•		•	x	•
Sau27	*agr*III ST88 V		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•		•		•
Sau30	*agr*II ST772 V	•		•				•	•	•	•	•		•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•				
Sau33	*agr*II ST772 V	•		•				•	•	•	•	•		•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•				
Sau22	*agr*II ST5 IV	•		•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	•
Sau31	*agr*II ST5 V	•		•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•				•
Sau15	*agr*III ST30 IV		•	•			•	•	•	•	•	•		•	•	•	•	•	•	•		•	•	•	•	•	•		x		•		•	•

Fig. 5 Presence of genes encoding miscellaneous virulence factors and adhesion factors in the CA-MRSA strains. The dots indicate positive results for probe hybridization and the Xs indicate ambiguous results. The function of each gene is listed in Supplementary Table S1 MSCRAMM, microbial surface components recognizing adhesive matrix molecules

the USA300 definition is based on a specific SmaI PFGE profile [21]. Although all USA300 strains initially shared this unique profile, some slightly divergent profiles were reported in the last few years, demonstrating the progressive diversification of this successful lineage [7]. All ST8 strains in our collection were related to the USA300 FPR3757 pulsotype, as assessed by a similarity percentage >80% (exactly, 82.38%). The pulsotype of Sau65 was the most closely related to the USA300 FPR3757 pulsotype (percentage of similarity=87.5%). Sau65 was also the only strain that harboured the ACME-specific *arcA* gene. The ACME element encodes proteins involved in an arginine deaminase pathway that converts L-arginine to carbon dioxide, ATP and ammonia. ACME has been proposed to play an important role in the pathogenesis of CA-MRSA skin infections, since arginine conversion raises the local pH, increasing the ability of USA300 to persist and spread on intact skin [9, 20]. However, some experimental data are not entirely consistent with this model [25]. The ACME element was rarely found in *S. aureus* clones other than USA300 [20]. On the basis of its characteristics, strain Sau65 can be considered to belong to the USA300 clone [9, 20, 21].

The other six ST8 strains that did not harbour the ACME-specific *arcA* gene shared all of the other characteristics of USA300 (ST, *spa*, PVL) that, according to Larsen et al., are even better markers than PFGE to assign a strain to the USA300 clone [26]. Therefore, the other six ST8 strains can be considered as an ACME-negative variant of the USA300 clone.

The antibiotic resistance profile of the USA300 strain was different from those of the ACME-negative variants. USA300 was multiantibiotic-resistant, since it harboured *msrA* and *mpbBM* (resistance to erythromycin), *aphA-3* (resistance to kanamycin), *sat* (resistance to streptothricin), *fosB* (resistance to fosfomycin) and, unique in our collection, also *mupR* (resistance to mupirocin). ACME-negative strains were more antibiotic-susceptible, bearing only the *fosB* gene. Strain Sau25 also harboured the *tetK* gene, but was phenotypically susceptible to tetracycline. Characteristically, ACME-negative strains harboured the *mer* operon (mercury resistance), which was not present in neither the USA300 isolate nor in the other CA-MRSA isolates examined. The *mer* operon is usually integrated within SCC*mec* type III, but it is likely that, in this case, it was carried by a plasmid, as previously described [27]. Both the USA300 and the ACME-

negative strains carried the enterotoxin cluster *entK-Q*. Sau17 and Sau18 were the only strains in our collection bearing an intact *hlb* (beta-haemolysin) gene; all of the other ST8 isolates harboured the truncated form due to the insertion of *sak*, *chp* and *scn* phage-borne genes. The profiles of the other virulence genes were similar in all ST8 strains, although *splE*, encoding the serine protease E, was absent in the USA300 strain and *bbp*, encoding the bone sialoprotein-binding protein, an adhesion factor, was variably present in the ST8 isolates. The microarray profile of the USA300 strain was similar to those previously reported for the USA300 lineage. Interestingly, the *mupR* gene has been rarely reported in USA300 isolates [23, 24, 28].

ST8 has been recognised as the major CA-MRSA clone also in other European countries, such as Austria, Bulgaria and Spain [10, 29]. In some studies, when additional typing was performed (e.g. ACME detection or PFGE), ST8 CA-MRSA isolates were identified as belonging to the USA300 clone [10].

2. *agr*III/ST80/SCC*mec*IV

   Four strains belonged to ST80, all of which carried the antibiotic resistance genes *aphA-3*, *sat*, *tetK* and *far* (resistance to fusidic acid). Strain Sau58 also carried *ermC*, encoding for erythromycin and clindamycin resistance. ST80 strains did not possess any enterotoxin genes or clusters. The gene *hlb* was truncated at the phage insertion site due to the presence of *sak* and *scn* genes. Microarray profiles were consistent with those previously published for ST80, showing that this clone is characterised by a specific antimicrobial resistance pattern and by specific virulence factors such as *etD* (encoding the exfoliative toxin D) and *edinB* (encoding for the epidermal cell differentiation inhibitor B), which are strong markers for the European ST80 clone [23, 24]. At variance with the vast majority of European countries, in Italy, ST80 appears not to be the most common CA-MRSA clone [10].

3. *agr*III/ST88/SCC*mec*V

   Two strains belonged to ST88. They possessed the bi-functional gene *aacA-aphD*, encoding gentamicin–kanamycin resistance. In this clone, enterotoxin P and the truncated *hlb* gene for the insertion of the *sak*, *scn* and *chp* genes were present. The *hsdS1* gene was found only in the two ST88 isolates. *hsdS1* encodes a site-specific deoxyribonuclease subunit type 1, involved in a restriction modification system responsible for DNA protection [30].

   CA-MRSA ST88 isolates are rather rare: they have been found only sporadically in Africa, Bangladesh, China and Europe [31, 32]. However, in Africa, the ST88 clone was found both in hospitals and in the community, both MSSA and MRSA [31]. Differently from the previously published microarray profile of this clone, ST88 isolates from this study did not bear *entA* or *tetK* genes [23].

4. *agr*II/ST772/SCC*mec*V

   Two strains belonged to ST772. They possessed the antibiotic resistance genes *msrA*, *mpbBM*, *aacA-aphD*, *aphA-3*, *sat* and *fosB*, and gave positive hybridisation for enterotoxins *entA*, *entK-Q* and *egc* (comprising *entG-I-M-N-O-U*) clusters and *scn*. The isolates lacked the leukocidins *lukD-lukE* and the serine proteases *splA*, *splB* and *splE*. Both isolates harboured the *cna* gene. This gene encodes a collagen-binding adhesin, a virulence factor that could have a role in necrotising pneumonia pathogenesis [33].

   ST772 isolates are rather rare: they have been found in Malaysia, Bangladesh, India and England [34, 35]. The toxin and virulence factor contents of the isolates were in accordance to those of the Bangladesh isolates [34], although our isolates did not carry *lukD* and *lukE* genes.

   ST772 is a single-locus variant of ST1. However, the microarray profile of ST772 is completely different from that of ST1, in terms of antibiotic resistance, toxin content, SCC*mec* elements and *agr* alleles [23, 24].

5. *agr*II/ST5/SCC*mec*IV–V

   Two strains shared this ST, but they harboured different SCC*mec* elements (IV and V). Uniquely among the isolates under study, the two ST5 isolates did not possess the *bla* genes encoding the staphylococcal penicillinase. The content of the other antibiotic resistance genes and the toxin genes was quite different in the two isolates. Sau22 possessed *fosB*, *dfrA* (trimethoprim resistance), *tetM* (tetracycline resistance) and *qacA*, responsible for quaternary ammonium compounds resistance tolerance. The enterotoxin cluster *egc* and the phage-borne genes *sak*, *scn* and *chp* were present. Sau31 harboured *aacA-aphD*, *tetK* and *fosB*, the enterotoxin gene *entP*, the enterotoxin clusters *entD-JR* and *egc*, and the phage-borne genes *sak* and *scn*. The two strains shared the same MSCRAMMs and proteases genes.

   The *agr*II/ST5 clone is widely disseminated and can present different gene profiles, as microarray results have previously described [23, 24].

6. *agr*III/ST30/SCC*mec*IV

   Only one strain belonged to ST30. This strain was relatively antibiotic-susceptible, since it possessed only the *fosB* gene. It harboured the enterotoxin *egc* cluster, *sak*, *scn*, *chp* and *cna* genes. As expected, in this strain, the allelic variants of some genes were found: for instance, the variant 2 of the leukocidin *lukY*, the alleles MRSA252 of *fib*, encoding a fibronectin-binding protein and of *isdA*, an immune-evasion factor. The

microarray profile is in general accordance with that of the ST30 strain MRSA252, with the exception that Sau15 lacks *aadD* and *ermA* [23]. ST30 corresponds to the South-West Pacific clone, a pandemic clone spread in Oceania, East-Asia, the United States, South America and some European countries [3].

Conclusions

Our data provide evidence of the diversity of the clones circulating in Italy. The presence of six distinct community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones probably reflects the introduction into the country of different clones by international travellers or immigrants. Among a limited number of Panton–Valentine leukocidin (PVL)-positive CA-MRSA isolates, we found isolates belonging to uncommon clones, such as *agr*III/ST88/SCC*mec*V and *agr*III/ST772/SCC*mec*V, which are only sporadically reported in other countries [31, 32, 34, 35].

Isolates belonging to ST8 were the most common. In previously published case reports [12, 13], some of the isolates were already assigned to the USA300 clone. In fact, with the exception of one strain that presented all of the USA300 characteristics, the other ST8 isolates should be defined as arginine catabolic mobile element (ACME)-negative USA300 strains. ACME-negative strains were identified inside the USA300 clone in the USA [7], Australia [28], Austria [10], Spain [29], Latin America [36] and Italy [37], but, often, they represented only a minority of the isolates. On the contrary, the majority of the ST8 isolates found in Italy are ACME-negative.

Microarray hybridisation has the great potentiality of investigating simultaneously the presence of a large number of genomic loci. Hence, it allows the detection of unexpected characteristics in a particular isolate that would not be searched for by more labourious methods (e.g. by polymerase chain reaction [PCR] assays). This applies to less common antibiotic resistance genes and to complex patterns of virulence genes. Some of the peculiarities found, if confirmed, have the potentiality to represent new epidemiological markers for the clones. One example is the *mer* operon, which, in our study, was recovered only in the ACME-negative USA300 strains and not in the ACME-positive USA300 or in the other CA-MRSA isolates. In the study by Monecke et al. [28], the *mer* operon was also present in the USA300 ACME-negative strains from Australia and not in the USA300 ACME-positive strains. Although the microarray has allowed to find characteristics that are unique to clones and strains, no specific patterns of toxin or virulence factors genes have been identified that characterise CA-MRSA or CA-MRSA strains causing specific diseases, such as necrotising pneumonia, as already pointed out by previous studies [23, 24, 28].

In conclusion, this study confirms that microarray hybridisation represents a valid alternative approach to the conventional molecular typing techniques, providing additional features that are complementary to the characterisation of the strains.

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References

1. Pantosti A, Sanchini A, Monaco M (2007) Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future Microbiol 2:323–334. doi:10.2217/17460913.2.3.323
2. Pantosti A, Venditti M (2009) What is MRSA? Eur Respir J 34:1190–1196. doi:10.1183/09031936.00007709
3. Deleo FR, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet 375:1557–1568. doi:10.1016/S0140-6736(09)61999-1
4. Higuchi W, Takano T, Teng LJ, Yamamoto T (2008) Structure and specific detection of staphylococcal cassette chromosome *mec* type VII. Biochem Biophys Res Commun 377:752–756. doi:10.1016/j.bbrc.2008.10.009
5. Shore AC, Rossney AS, Kinnevey PM, Brennan OM, Creamer E, Sherlock O, Dolan A, Cunney R, Sullivan DJ, Goering RV, Humphreys H, Coleman DC (2010) Enhanced discrimination of highly clonal ST22-methicillin-resistant *Staphylococcus aureus* IV isolates achieved by combining *spa*, *dru*, and pulsed-field gel electrophoresis typing data. J Clin Microbiol 48:1839–1852. doi:10.1128/JCM.02155-09
6. Dauwalder O, Lina G, Durand G, Bes M, Meugnier H, Jarlier V, Coignard B, Vandenesch F, Etienne J, Laurent F (2008)

Epidemiology of invasive methicillin-resistant *Staphylococcus aureus* clones collected in France in 2006 and 2007. J Clin Microbiol 46:3454–3458. doi:10.1128/JCM.01050-08

7. McDougal LK, Fosheim GE, Nicholson A, Bulens SN, Limbago BM, Shearer JE, Summers AO, Patel J (2010) Emergence of resistance among USA300 methicillin-resistant *Staphylococcus aureus* isolates causing invasive disease in the United States. Antimicrob Agents Chemother 54:3804–3811. doi:10.1128/AAC.00351-10
8. Boyle-Vavra S, Daum RS (2007) Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton–Valentine leukocidin. Lab Invest 87:3–9. doi:10.1038/labinvest.3700501
9. Diep BA, Stone GG, Basuino L, Graber CJ, Miller A, des Etages SA, Jones A, Palazzolo-Ballance AM, Perdreau-Remington F, Sensabaugh GF, DeLeo FR, Chambers HF (2008) The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J Infect Dis 197:1523–1530. doi:10.1086/587907
10. Otter JA, French GL (2010) Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Europe. Lancet Infect Dis 10:227–239. doi:10.1016/S1473-3099(10)70053-0
11. Monaco M, Antonucci R, Palange P, Venditti M, Pantosti A (2005) Methicillin-resistant *Staphylococcus aureus* necrotizing pneumonia. Emerg Infect Dis 11:1647–1648
12. Valentini P, Parisi G, Monaco M, Crea F, Spanu T, Ranno O, Tronci M, Pantosti A (2008) An uncommon presentation for a severe invasive infection due to methicillin-resistant *Staphylococcus aureus* clone USA300 in Italy: a case report. Ann Clin Microbiol Antimicrob 7:11. doi:10.1186/1476-0711-7-11
13. Tinelli M, Pantosti A, Lusardi C, Vimercati M, Monaco M (2007) First detected case of community-acquired methicillin-resistant *Staphylococcus aureus* skin and soft tissue infection in Italy. Euro Surveill 12:E070412.1
14. Marchese A, Gualco L, Maioli E, Debbia E (2009) Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int J Antimicrob Agents 34:424–428. doi:10.1016/j.ijantimicag.2009.06.016
15. Stefani S, Bongiorno D, Cafiso V, Campanile F, Crapis M, Cristini F, Sartor A, Scarparo C, Spina D, Viale P (2009) Pathotype and susceptibility profile of a community-acquired methicillin-resistant *Staphylococcus aureus* strain responsible for a case of severe pneumonia. Diagn Microbiol Infect Dis 63:100–104. doi:10.1016/j.diagmicrobio.2008.09.012
16. European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2010) Clinical breakpoints. Available online at: http://www.eucast.org/eucast_disk_diffusion_test/breakpoints/
17. Clinical and Laboratory Standards Institute (CLSI) (2008) Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement. M100-S18. Vol. 28; No. 1. CLSI, Wayne, PA
18. The British Society for Antimicrobial Chemotherapy (BSAC) (2010) Clinical breakpoints. Available online at: http://www.bsac.org.uk/Susceptibility+Testing/Breakpoints
19. Monaco M, Sanchini A, Grundmann H, Pantosti A, EARSS-Italy S. aureus typing Group (2010) Vancomycin-heteroresistant phenotype in invasive methicillin-resistant *Staphylococcus aureus* isolates belonging to *spa* type 041. Eur J Clin Microbiol Infect Dis 29:771–777. doi:10.1007/s10096-010-0922-2
20. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 367:731–739. doi:10.1016/S0140-6736(06)68231-7
21. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41:5113–5120
22. van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, Fussing V, Green J, Feil E, Gerner-Smidt P, Brisse S, Struelens M, European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group on Epidemiological Markers (ESGEM) (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13(Suppl 3):S1–S46. doi:10.1111/j.1469-0691.2007.01786.x
23. Monecke S, Slickers P, Ehricht R (2008) Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol 53:237–251. doi:10.1111/j.1574-695X.2008.00426.x
24. Monecke S, Berger-Bächi B, Coombs G, Holmes A, Kay I, Kearns A, Linde HJ, O'Brien F, Slickers P, Ehricht R (2007) Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton–Valentine leukocidin. Clin Microbiol Infect 13:236–249. doi:10.1111/j.1469-0691.2006.01635.x
25. Montgomery CP, Boyle-Vavra S, Daum RS (2009) The arginine catabolic mobile element is not associated with enhanced virulence in experimental invasive disease caused by the community-associated methicillin-resistant *Staphylococcus aureus* USA300 genetic background. Infect Immun 77:2650–2656. doi:10.1128/IAI.00256-09
26. Larsen AR, Goering R, Stegger M, Lindsay JA, Gould KA, Hinds J, Sørum M, Westh H, Boye K, Skov R (2009) Two distinct clones of methicillin-resistant *Staphylococcus aureus* (MRSA) with the same USA300 pulsed-field gel electrophoresis profile: a potential pitfall for identification of USA300 community-associated MRSA. J Clin Microbiol 47:3765–3768. doi:10.1128/JCM.00934-09
27. Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K (2001) Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 45:1323–1336. doi:10.1128/AAC.45.5.1323-1336.2001
28. Monecke S, Ehricht R, Slickers P, Tan HL, Coombs G (2009) The molecular epidemiology and evolution of the Panton–Valentine leukocidin-positive, methicillin-resistant *Staphylococcus aureus* strain USA300 in Western Australia. Clin Microbiol Infect 15:770–776. doi:10.1111/j.1469-0691.2009.02792.x
29. Blanco R, Tristan A, Ezpeleta G, Larsen AR, Bes M, Etienne J, Cisterna R, Laurent F (2011) Molecular epidemiology of Panton–Valentine leukocidin-positive *Staphylococcus aureus* in Spain: emergence of the USA300 clone in an autochthonous population. J Clin Microbiol 49:433–436. doi:10.1128/JCM.02201-10
30. Wilson GG (1991) Organization of restriction-modification systems. Nucleic Acids Res 19:2539–2566
31. Breurec S, Zriouil SB, Fall C, Boisier P, Brisse S, Djibo S, Etienne J, Fonkoua MC, Perrier-Gros-Claude JD, Pouillot R, Ramarokoto CE, Randrianirina F, Tall A, Thiberge JM; Working Group on *Staphylococcus aureus* infections, Laurent F, Garin B (2011) Epidemiology of methicillin-resistant *Staphylococcus aureus* lineages in five major African towns: emergence and spread of atypical clones. Clin Microbiol Infect 17:160–165. doi:10.1111/j.1469-0691.2010.03219.x

32. Yao D, Yu FY, Qin ZQ, Chen C, He SS, Chen ZQ, Zhang XQ, Wang LX (2010) Molecular characterization of *Staphylococcus aureus* isolates causing skin and soft tissue infections (SSTIs). BMC Infect Dis 10:133. doi:10.1186/1471-2334-10-133
33. de Bentzmann S, Tristan A, Etienne J, Brousse N, Vandenesch F, Lina G (2004) *Staphylococcus aureus* isolates associated with necrotizing pneumonia bind to basement membrane type I and IV collagens and laminin. J Infect Dis 190:1506–1515. doi:10.1086/424521
34. D'Souza N, Rodrigues C, Mehta A (2010) Molecular characterization of methicillin-resistant *Staphylococcus aureus* with emergence of epidemic clones of sequence type (ST) 22 and ST 772 in Mumbai, India. J Clin Microbiol 48:1806–1811. doi:10.1128/JCM.01867-09
35. Ellington MJ, Ganner M, Warner M, Cookson BD, Kearns AM (2010) Polyclonal multiply antibiotic-resistant methicillin-resistant *Staphylococcus aureus* with Panton–Valentine leucocidin in England. J Antimicrob Chemother 65:46–50. doi:10.1093/jac/dkp386
36. Reyes J, Rincón S, Díaz L, Panesso D, Contreras GA, Zurita J, Carrillo C, Rizzi A, Guzmán M, Adachi J, Chowdhury S, Murray BE, Arias CA (2009) Dissemination of methicillin-resistant *Staphylococcus aureus* USA300 sequence type 8 lineage in Latin America. Clin Infect Dis 49:1861–1867. doi:10.1086/648426
37. Baldan R, Tassan Din C, Semeraro G, Costa C, Cichero P, Scarpellini P, Moro M, Cirillo DM (2009) Severe community-onset infections in healthy individuals caused by community-acquired MRSA in an Italian teaching hospital, 2006–2008. J Hosp Infect 72:271–273. doi:10.1016/j.jhin.2009.04.007

ANNEXE VI

Methicillin-Resistant *Staphylococcus capitis* with Reduced Vancomycin Susceptibility Causes Late-Onset Sepsis in Intensive Care Neonates

Rasigade JP, Raulin O, Picaud JC, Tellini C, Bes M, Grando J, Ben Saïd M, Claris O, Etienne J, Tigaud S, Laurent F

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Methicillin-Resistant *Staphylococcus capitis* with Reduced Vancomycin Susceptibility Causes Late-Onset Sepsis in Intensive Care Neonates

Jean-Philippe Rasigade[1,2*], Olivia Raulin[2], Jean-Charles Picaud[3], Charlotte Tellini[2], Michele Bes[1], Jacqueline Grando[4], Mohamed Ben Saïd[3], Olivier Claris[5], Jerome Etienne[1], Sylvestre Tigaud[2], Frederic Laurent[1,2]

1 INSERM U851, National Reference Center for Staphylococci, University of Lyon, Lyon, France, **2** Department of Clinical Microbiology, Northern Hospital Group, Hospices Civils de Lyon, Lyon, France, **3** Department of Neonatology, Northern Hospital Group, Hospices Civils de Lyon, Lyon, France, **4** Department of Hygiene and Epidemiology, Edouard Herriot Hospital Center, Hospices Civils de Lyon, Lyon, France, **5** Department of Neonatology, Eastern Hospital Group, Hospices Civils de Lyon, Lyon, France

Abstract

Background: Coagulase-negative staphylococci, mainly *Staphylococcus epidermidis*, are the most frequent cause of late-onset sepsis (LOS) in the neonatal intensive care unit (NICU) setting. However, recent reports indicate that methicillin-resistant, vancomycin-heteroresistant *Staphylococcus capitis* could emerge as a significant pathogen in the NICU. We investigated the prevalence, clonality and vancomycin susceptibility of *S. capitis* isolated from the blood of NICU infants and compared these data to adult patients.

Methodology/Principal Findings: We conducted a retrospective laboratory-based survey of positive blood cultures in NICU infants ≥3 days of age (n = 527) and in adult ICU patients ≥18 years of age (n = 1473) who were hospitalized from 2004 to 2009 in two hospital centers in Lyon, France. *S. capitis* was the most frequent pathogen in NICU infants, ahead of *S. epidermidis* (39.1% vs. 23.5% of positive blood cultures, respectively). Conversely, *S. capitis* was rarely found in adult ICU patients (1.0%) compared to *S. epidermidis* (15.3%). *S. capitis* bloodstream isolates were more frequently resistant to methicillin when collected from NICU infants than from adult patients (95.6% vs. 53.3%, respectively). Furthermore, we collected and characterized 53 *S. capitis* bloodstream isolates from NICU infants and adult patients from six distant cities. All methicillin-resistant *S. capitis* isolates from NICU infants were clonally related as determined by pulsed-field gel electrophoresis. These isolates harbored a type V-related staphylococcal chromosomal cassette *mec* element, and constantly showed either vancomycin resistance (37.5%) or heteroresistance (62.5%). Conversely, the isolates that were collected outside of the NICU were genetically diverse and displayed much lower rates of vancomycin resistance and heteroresistance (7.7% and 23.1%, respectively).

Conclusions/Significance: A clonal population of methicillin-resistant *S. capitis* strains has spread into several French NICUs. These isolates exhibit reduced susceptibility to vancomycin, which is the most widely used antimicrobial agent in the NICU setting.

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* E-mail: jean-philippe.rasigade@chu-lyon.fr

Introduction

Constant improvements in neonatal intensive care have led to better survival rates for very-low-birth-weight infants (VLBW, <1500 g), but the high incidence of nosocomial, late-onset sepsis (LOS, occurring after 3 days of age) is still a leading cause of morbidity and mortality [1,2]. Coagulase-negative staphylococci (CoNS) are the most frequently encountered pathogens in bloodstream infections in neonatal intensive care units (NICUs) [3,4]. Several risk factors for such infections have been identified, including low gestational age, low birth weight [5], parenteral nutrition with intravenous lipids, and the presence of a central venous catheter [6,7]. Although CoNS bloodstream infections are significantly less severe than those caused by other pathogens [4], they lead to prolonged hospitalization and increased antibiotic use [4,8].

Staphylococcus epidermidis has been shown to be the predominant species in CoNS bacteremia in both adult and pediatric patients [9–12]; in the NICU setting, it represents 70% of CoNS isolates [12–15]. Among non-*epidermidis* CoNS, *Staphylococcus capitis* is rarely, if ever, isolated from bacteremic adult patients [16]. Although this species has been occasionally reported in infective endocarditis cases [17,18], it is most often considered to be a blood culture contaminant [16]. However, in the NICU setting, recent

studies have indicated that methicillin-resistant *S. capitis* could emerge as a significant pathogen, causing LOS in VLBW infants [14,19–22]. The high prevalence of methicillin-resistant CoNS in NICUs usually leads to frequent vancomycin use. Concerns have been raised about the spread of vancomycin-heteroresistant *S. capitis* strains in NICUs and their involvement in persistent bacteremia despite prolonged vancomycin therapy [19,20,23].

The unusually high prevalence of methicillin-resistant *S. capitis* bloodstream infections in the NICUs of the University Hospital of Lyon, France, prompted this study. The objectives were: (1) to describe the species distribution of bloodstream CoNS isolates in these NICUs; (2) to investigate the clonality of *S. capitis* that causes LOS in the NICUs of Lyon, and compare it to *S. capitis* strains found in adult patients and in other French NICUs; and (3) to investigate vancomycin resistance and heteroresistance in these strains.

Methods

Retrospective prevalence study

We conducted a retrospective laboratory-based survey of blood cultures at two NICUs located in two different hospital centers within the University Hospital of Lyon, France. Both NICUs provide third-level neonatal care, with approximately 70% (Northern Hospital Group) and 55% (Eastern Hospital Group) of infants having VLBW. The inclusion period was from January 1, 2004 to December 31, 2009. The microbiological records of blood cultures drawn from NICU infants after the third day of life (to exclude early-onset sepsis) were obtained from the computerized databases of the clinical microbiology laboratories of each hospital. For comparison purposes, the blood culture results of patients over 18 years of age who were hospitalized in the ICUs of these hospitals during the study period were also reviewed. The first positive blood culture result from each patient was considered for analysis. We also determined the number of patients who had different blood cultures positive with different pathogens. Both participating microbiology laboratories performed species-level identification of the bacterial isolates and antimicrobial susceptibility testing (AST) with the automated BD Phoenix system (Becton Dickinson, Sparks, MD). The AST results were interpreted according to the recommendations of the French Society for Microbiology [24].

Because the study was laboratory-based and no clinical data were obtained, CoNS-positive blood cultures were interpreted to be probable or possible CoNS bacteremia based on the number of positive blood cultures and the patient setting (i.e., NICU infants or adult ICU patients). In both NICU infants and adult ICU patients, probable CoNS bacteremia was defined as ≥2 blood culture sets positive with the same CoNS species within a 3-day period [25]. CoNS-positive blood cultures in adult ICU patients that did not match these criteria were excluded (n = 585). However, because repeated blood cultures are not frequently performed in NICU infants compared to adult patients, single CoNS-positive blood cultures in NICU infants were interpreted as possible CoNS bacteremia and included for analysis. Seventeen CoNS-positive blood cultures were excluded because they were explicitly recorded as false positives in the microbiological record, and thus not identified at the species level. Finally, we excluded blood cultures that were positive with organisms considered to be contaminants other than CoNS, including *Bacillus* spp. (n = 4 NICU infants and 10 ICU patients) and *Micrococcus* spp. (n = 1 NICU infant and 11 ICU patients).

The resistance patterns of *S. capitis* to penicillin, methicillin, gentamicin, erythromycin, clindamycin, pristinamycin, rifampin, fusidic acid and fluoroquinolones were recorded and compared between NICU and adult ICU isolates. Two records were excluded because AST results were incomplete and the corresponding isolates could not be recovered from the strain collection of the microbiology laboratory. The results regarding vancomycin susceptibility were excluded from the analysis because the ≤4 mg/L clinical breakpoint used during the 2004–2009 inclusion period differed from the ≤2 mg/L clinical breakpoint recommended since 2010 by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [26]. Because the study consisted of a retrospective review of routine microbiological data that were analyzed anonymously, approval by the ethics committee and informed consent were not required.

S. capitis isolates

To investigate the population structure of *S. capitis* at the regional level, *S. capitis* isolates were collected retrospectively among routinely stored clinical strains from participating centers in distant cities spanning the French territory, namely Caen, Limoges, Lyon, Saint-Etienne, Troyes and Versailles. In each laboratory, all bloodstream isolates with the exception of isolates interpreted as contaminants were stored regardless of their resistance profile, according to the French guidelines on bacterial strain storage [27]. *S. capitis* bloodstream isolates from NICU infants and adult/older pediatric patients regardless of their methicillin resistance status were included. The inclusion period was from January 2006 to April 2009 for the isolates collected from the Northern Hospital Group of Lyon, and from January 2008 to April 2009 for the isolates collected from the other participating centers because of the shorter duration of routine storage of clinical strains in these laboratories. Because the strain collection at Lyon consisted of a large number of *S. capitis* NICU isolates (n = 125), selection was restricted to 25 isolates using a random sampling algorithm without replacement by means of the XLStat software version 7.5.2 (Addinsoft SARL, Paris, France). Additionally, an *S. capitis* strain that was involved in a previously reported *S. capitis* outbreak at the NICU of Nantes from 2000 to 2003 was kindly provided by J. Caillon and also included in the study [22]. A total of 53 isolates were investigated, including 40 isolates from NICU infants (Lyon, n = 25; Limoges, n = 7; Saint-Etienne, n = 3; Caen, n = 2; Troyes, n = 2; and Nantes, n = 1) and 13 isolates from adult/older pediatrics patients (Lyon, n = 12; and Versailles, n = 1). Species-level identification of all *S. capitis* isolates included in this study was confirmed by amplification and sequence analysis of the *tuf* gene as previously described [28].

Pulsed-field gel electrophoresis

All *S. capitis* isolates (n = 53) were characterized by PFGE as previously described [29]. Genomic DNA was digested with *Sma*I restriction endonuclease, which discriminates well between staphylococci, including *S. capitis* [30]. After staining with ethidium bromide, electrophoresis gels were photographed and analyzed with GelCompar software version 4.1 (Applied Math NV, Saint-Martens-Latem, Belgium) using the unweighted pair group method with arithmetic mean (UPGMA). The Dice correlation coefficient was used to determine the similarity percentage between DNA restriction patterns, which were assigned to pulsotypes using >80% similarity with a band position tolerance of 1% as previously described [31]. The genetic diversity of the isolates from NICU infants was compared with the isolates from adult/older pediatric patients using Simpson's index of diversity (SID), which is defined as the percentage probability that two isolates chosen at random are unrelated [32].

Methicillin resistance and staphylococcal chromosomal cassette *mec* typing

The susceptibility of the 53 selected isolates to oxacillin and cefoxitin was determined by the standard agar diffusion technique as recommended by the French Society for Microbiology [24]. SCC*mec* typing was performed on a subset of 23 methicillin-resistant isolates representative of each pulsotype and geographic origin. Multiplex PCRs were performed to determine the allelic variants of both the chromosomal recombinase *ccr* gene complex cassette and the *mec* locus according to the method established by Kondo et al. [33]. Each combination of *ccr* and *mec* alleles was related to an SCC*mec* type when applicable, following the recommendations of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) [34].

Vancomycin resistance and heteroresistance testing

Vancomycin minimum inhibitory concentrations (MICs) were determined using the E-Test method (AB Biodisk, Solna, Sweden) with a McFarland density 0.5 inoculum on Mueller-Hinton agar as recommended by the manufacturer. The E-Test method was preferred to the CLSI broth microdilution reference method because of its better performance at detecting staphylococci with reduced vancomycin susceptibility [35,36]. Strains exhibiting a vancomycin MIC>2 mg/L were considered to be resistant according to EUCAST breakpoints [26]. Strains with a vancomycin MIC≤2 mg/L were tested for vancomycin heteroresistance using the brain heart infusion (BHI) screen agar method as previously described [37]. Briefly, an overnight blood agar plate culture was suspended in 0.9% saline and adjusted to McFarland 0.5 turbidity. Four 10-μL droplets of the suspension were dropped using a pipette onto a BHI agar plate (Difco, Becton Dickinson) containing 16 g/L pancreatic digest of casein (Bacto, Becton Dickinson) and 4 μg/mL vancomycin. Plates were incubated at 35°C and the colonies in each droplet were counted after 48 h. A strain was considered to be heteroresistant to vancomycin if ≥1 droplet had ≥2 colonies. The vancomycin-susceptible *S. aureus* strain ATCC 29213 was used as a negative control [37]. The vancomycin-heteroresistant *S. aureus* strain Mu3 and the vancomycin-resistant *S. aureus* strain Mu50 were used as positive controls [38]. The results from each experiment were recorded only when positive and negative controls were confirmed.

Statistical analysis

The differences in the prevalence of each bacterial species in the positive blood cultures between patient groups and in the antimicrobial resistance rates were analyzed using a two-tailed Fisher's exact test. *P* values were corrected for multiple testing using the Holm-Bonferroni method where appropriate. The differences in vancomycin MICs were analyzed using a non-parametric two-tailed Mann-Whitney *U*-test. *P* values of ≤0.05 were considered to be statistically significant. All statistical tests were performed using the XLStat software version 7.5.2.

Results

Methicillin-resistant *S. capitis* is the leading cause of LOS in NICU infants

In total, 2,628 patients with positive blood cultures were identified from the laboratory databases, and 2,000 cases were included in the final analysis (Fig. 1). Blood cultures were more likely to grow CoNS when drawn from NICU infants than from adult ICU patients ($P<0.001$, two-tailed Fisher's exact test with Holm-Bonferroni correction; Table 1). *S. capitis* was the most prevalent pathogen that caused bloodstream infections in NICU infants (39.1%) but accounted for only 1.0% of positive blood cultures from adult ICU patients ($P<0.001$). Overall, 54 out of 206 (26.2%) *S. capitis*-positive blood cultures from NICU infants fulfilled the definition for probable CoNS sepsis (≥2 blood culture sets were positive with the same species within a 3-day period), and 152 (73.8%) blood cultures were thus considered to be possible *S. capitis* sepsis. These proportions did not differ significantly from the non-*capitis* CoNS-positive blood cultures (probable sepsis, n = 41/210, 19.5%; possible sepsis, n = 169/210, 80.5%; $P = 0.13$). There was no significant evolution of *S. capitis* prevalence from 2004 to 2009 in either group of patients (data not shown).

The overall proportion of patients who had ≥2 blood cultures positive with ≥2 different pathogens was significantly lower in NICU infants than adult ICU patients (n = 177/527, 33.6%, vs. n = 960/1473, 65.2%, respectively; $P<0.001$). Among patients whose first positive blood culture grew *S. capitis*, NICU infants were significantly less likely than adult ICU patients to have subsequent blood cultures positive with another pathogen (n = 49/206, 23.8%, vs. n = 8/15, 53.3%, respectively; $P = 0.03$). Finally, among patients whose first positive blood culture grew a pathogen other than *S. capitis*, NICU infants were significantly more likely than adult ICU patients to have subsequent blood cultures positive with *S. capitis* (n = 42/321, 13.1%, vs. n = 16/1458, 1.1%, respectively; $P<0.001$).

The antimicrobial resistance patterns of *S. capitis* bloodstream isolates from NICU infants (n = 204) and adult ICU patients (n = 15) were compared. The isolates exhibited specific antimicrobial susceptibility patterns depending on the patient setting (Table 2). NICU isolates were more frequently resistant to beta-lactams and gentamicin ($P<0.001$) than adult ICU isolates and more frequently susceptible to erythromycin and fluoroquinolones ($P<0.05$). Among the NICU isolates, the methicillin-resistant isolates were more frequently resistant to gentamicin ($P<0.001$), and more frequently susceptible to fusidic acid than were methicillin-susceptible isolates ($P<0.05$; Table 3).

S. capitis isolates from distant NICUs are clonal and harbor a type V-related SCC*mec* element

Of 53 selected isolates, 49 (92.5%) were resistant to methicillin, including 39 NICU isolates (97.5%) and 10 non-NICU isolates (76.9%). PFGE identified 13 different pulsotypes, designated as NRCS-A to -M (Fig. 2). Except for a single methicillin-susceptible isolate, all NICU isolates that were collected from 7 different NICUs in 6 different cities belonged to the same pulsotype (NRCS-A). In contrast, the 14 remaining isolates, including 1 methicillin-susceptible NICU isolate and 13 isolates from adult or older pediatric patients, belonged to 13 different pulsotypes (NRCS-A to -M). The only NRCS-A isolate that was not from a NICU infant was collected from a 4-year-old patient who was hospitalized in the pediatric ICU of Lyon. Simpson's index of diversity was 4.9% (95% CI, 0.0–14.3%) for the NICU isolates and 91.1% (95% CI, 88.0–94.2%) for the adult/older pediatric isolates.

SCC*mec* typing was applied to a subset of 23 methicillin-resistant *S. capitis* isolates representative of each pulsotype and geographic origin, and all NRCS-A isolates were found to share the *mec* complex C and *ccr* C genes, whose combination matches the features of SCC*mec* type V (Fig. 2) [34]. The same *mec* complex/*ccr* gene combination was only found in one pulsotype NRCS-G isolate from an adult patient from Versailles. The 7 remaining methicillin-resistant isolates from adult patients belonged to pulsotypes NRCS-D, -E, and -H to -L and were found to harbor

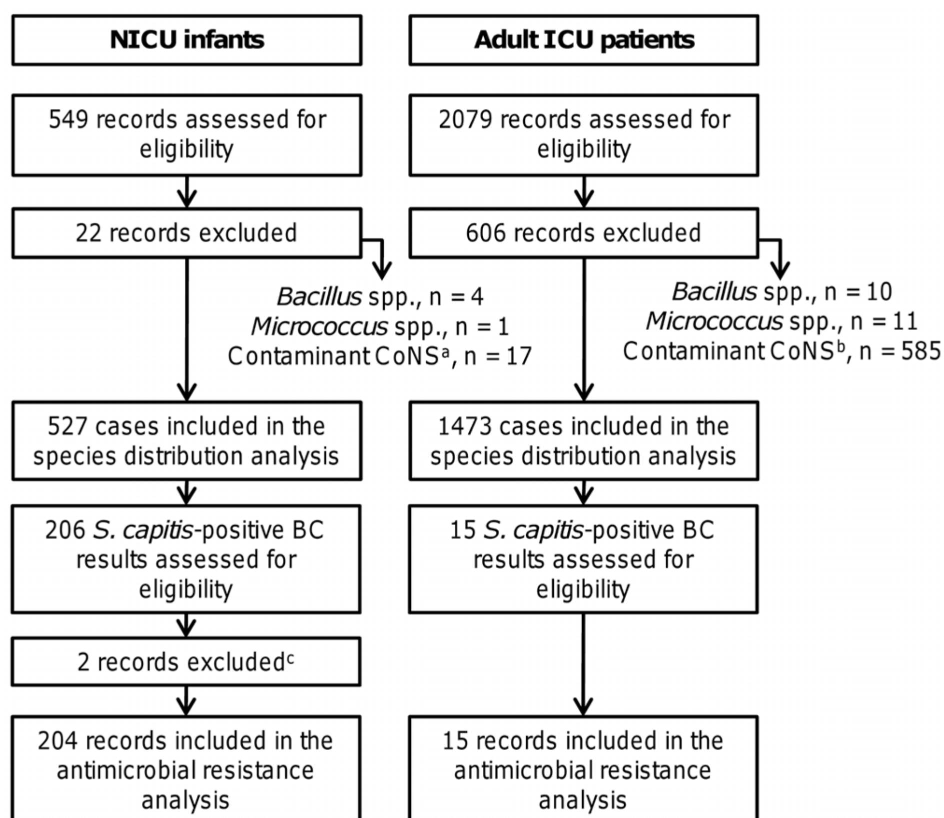

Figure 1. Flow diagram of case selection. (N)ICU, (neonatal) intensive care unit; CoNS, coagulase-negative staphylococci; BC, blood culture. [a]In NICU infants, a single CoNS-positive blood culture was interpreted as possible bacteremia and included in the analysis. The CoNS-positive results that were excluded were explicitly recorded as a contaminant in the microbiology record. [b]In adult patients, a single CoNS-positive blood culture was interpreted as a contaminant and excluded from the analysis. [c]Records were excluded when antimicrobial susceptibility results were not available.
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either the *mec* complex B/*ccr* A2B2 (matching SCC*mec* IV) or the *mec* complex C/*ccr* A2B2C (a combination that has not yet been assigned to an SCC*mec* type to our knowledge).

NRCS-A isolates have reduced vancomycin susceptibility

The mean vancomycin MIC of NRCS-A isolates was significantly higher (2.8 mg/L, range 1.5–12 mg/L) than the NRCS-B to -M isolates (1.7 mg/L, range 1–4 mg/L; $P<0.01$, two-tailed Mann-Whitney *U*-test) (Fig. 3). Regarding vancomycin susceptibility, as interpreted using the EUCAST 2010 recommendations [39], the proportion of vancomycin-resistant isolates was 4.9-fold higher in pulsotype NRCS-A than in the other pulsotypes (37.5% vs. 7.7%, respectively; $P = 0.08$, Fisher's exact test). Moreover, the proportion of vancomycin-heteroresistant isolates was 2.7-fold higher in NRCS-A than in the other pulsotypes (62.5% vs. 23.1%, respectively; $P<0.05$). All NRCS-A isolates with

Table 1. A species distribution comparison of *Staphylococcus* spp. isolates from the positive blood cultures of patients in neonatal intensive care units (NICUs) and adult ICUs, 2004–2009.

	No. (%) of blood cultures (one per patient)			
Blood culture result	**NICU infants**	**Adult ICU patients**	***P* value[a]**	**Odds ratio (95% CI)**
Total	527	1473		
S. aureus	65 (12.3)	166 (11.3)	0.525	1.11 (0.82–1.50)
Coagulase-negative staphylococci	416 (78.9)	331 (22.5)	<0.001	12.9 (10.1–16.5)
S. epidermidis	124 (23.5)	226 (15.3)	<0.001	1.70 (1.33–2.17)
S. capitis	206 (39.1)	15 (1.0)	<0.001	62.4 (36.4–106.8)
Other CoNS species	86 (16.3)	90 (6.1)	<0.001	3.00 (2.19–4.10)

[a]The differences between the groups were tested for statistical significance using a two-tailed Fisher's exact test. *P* values were corrected for multiple testing using the Holm-Bonferroni method.
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Table 2. A retrospective comparison of the antimicrobial resistance profiles of *Staphylococcus capitis* bloodstream isolates from patients in neonatal intensive care units (NICUs) and adult ICUs, 2004–2009.

	No. (%) of resistant isolates			
Antimicrobial agent	**NICU isolates (n = 204)**	**Adult ICU isolates (n = 15)**	***P* value[a]**	**Odds ratio (95% CI)**
Penicillin	202 (99.0)	10 (66.7)	<0.001	50.5 (8.70–293.1)
Methicillin	195 (95.6)	8 (53.3)	<0.001	19.0 (5.63–63.9)
Gentamicin	194 (95.1)	3 (20.0)	<0.001	77.6 (18.8–319.7)
Erythromycin	29 (14.2)	7 (46.7)	0.022	0.19 (0.06–0.56)
Clindamycin	21 (10.3)	4 (26.7)	0.227	0.32 (0.09–1.08)
Pristinamycin	13 (6.4)	1 (6.7)	1.000	0.95 (0.12–7.82)
Rifampin	91 (44.6)	3 (20.0)	0.205	3.22 (0.88–11.8)
Fusidic acid	11 (5.4)	4 (26.7)	0.050	0.16 (0.04–0.57)
Fluoroquinolones	10 (4.9)	7 (46.7)	<0.001	0.06 (0.02–0.20)

[a]The differences between the groups were tested for statistical significance using a two-tailed Fisher's exact test. *P* values were corrected for multiple testing using the Holm-Bonferroni method.

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a vancomycin MIC in the susceptible range were heteroresistant. Finally, the proportion of isolates exhibiting either vancomycin resistance or heteroresistance was significantly higher in pulsotype NRCS-A than in the other pulsotypes (100.0% vs. 30.8%, respectively; $P<0.001$).

Discussion

Several major findings emerged from this retrospective study of 2,000 positive blood cultures from NICU infants and adult ICU patients from 2004 to 2009 and from the molecular characterization of a panel of *S. capitis* clinical isolates. First, methicillin-resistant *S. capitis* was the most prevalent pathogen that caused LOS in the NICUs of Lyon, France, whereas this species was rarely isolated from the blood of adult ICU patients. Second, all methicillin-resistant *S. capitis* bloodstream isolates collected from several NICUs spanning the French territory belonged to the same pulsotype, which was designated as pulsotype NRCS-A, whereas the methicillin-susceptible isolates and the isolates from adult and older pediatric patients exhibited high genetic diversity. Third, pulsotype NRCS-A isolates constantly showed either vancomycin resistance (37.5%) or heteroresistance (62.5%), whereas these rates were 4.9- and 2.7-fold, respectively, lower in the isolates collected outside of the NICU. Collectively, these findings indicate that a clonal population of methicillin-resistant *S. capitis* exhibiting reduced susceptibility to vancomycin has spread into several NICUs in France.

S. capitis has been sporadically incriminated in NICU outbreaks [22], but the prevalence of this pathogen as a cause of LOS in French NICUs had not been investigated prior to this study. Several surveillance programs, such as RAISIN and NEOCAT [40,41], have focused on healthcare-associated bloodstream infections in French ICUs and NICUs. However, *S. capitis* prevalence could not be inferred from the results of these studies because CoNS prevalence was not reported at the species level.

Table 3. A retrospective comparison of the antimicrobial resistance profiles of methicillin-resistant and methicillin-susceptible *Staphylococcus capitis* bloodstream isolates from patients in neonatal intensive care units, 2004–2009.

	No. (%) of resistant isolates			
Antimicrobial agent	**Methicillin-resistant isolates (n = 195)**	**Methicillin-susceptible isolates (n = 9)**	***P* value[a]**	**Odds ratio (95% CI)**
Penicillin	195 (100.0)	7 (77.8)	<0.001	NC[b]
Gentamicin	192 (98.5)	2 (22.2)	<0.001	224.0 (32.1–1561.3)
Erythromycin	27 (13.8)	2 (22.2)	1.000	0.56 (0.11–2.85)
Clindamycin	21 (10.8)	0 (0.0)	1.000	NC
Pristinamycin	13 (6.7)	0 (0.0)	1.000	NC
Rifampin	89 (45.6)	2 (22.2)	0.966	2.94 (0.60–14.5)
Fusidic acid	8 (4.1)	3 (33.3)	0.049	0.09 (0.02–0.41)
Fluoroquinolones	10 (5.1)	0 (0.0)	1.000	NC

[a]The differences between the groups were tested for statistical significance using a two-tailed Fisher's exact test. *P* values were corrected for multiple testing using the Holm-Bonferroni method.
[b]NC, not calculable.

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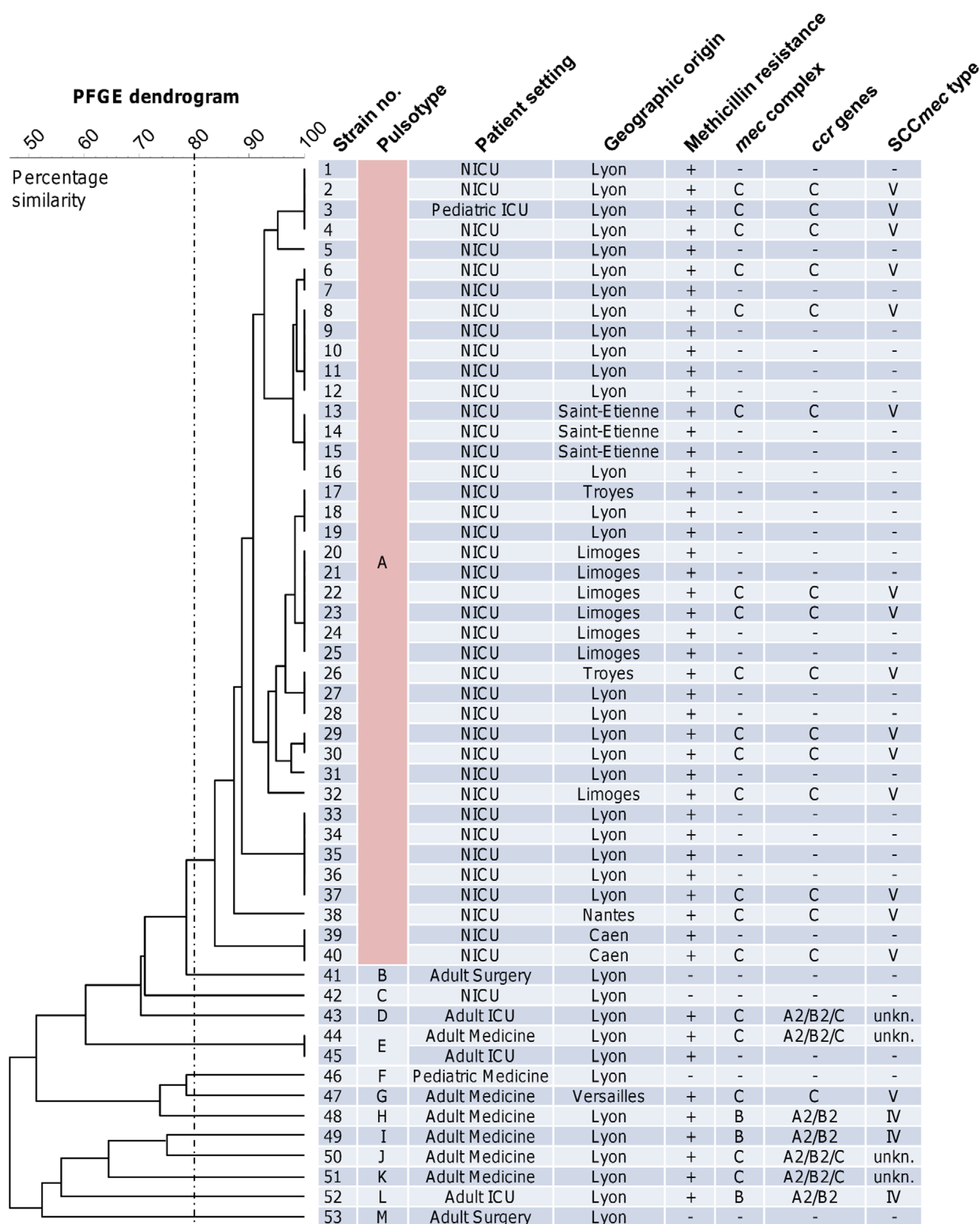

Strain no.	Pulsotype	Patient setting	Geographic origin	Methicillin resistance	*mec* complex	*ccr* genes	SCC*mec* type
1	A	NICU	Lyon	+	-	-	-
2		NICU	Lyon	+	C	C	V
3		Pediatric ICU	Lyon	+	C	C	V
4		NICU	Lyon	+	C	C	V
5		NICU	Lyon	+	-	-	-
6		NICU	Lyon	+	C	C	V
7		NICU	Lyon	+	-	-	-
8		NICU	Lyon	+	C	C	V
9		NICU	Lyon	+	-	-	-
10		NICU	Lyon	+	-	-	-
11		NICU	Lyon	+	-	-	-
12		NICU	Lyon	+	-	-	-
13		NICU	Saint-Etienne	+	C	C	V
14		NICU	Saint-Etienne	+	-	-	-
15		NICU	Saint-Etienne	+	-	-	-
16		NICU	Lyon	+	-	-	-
17		NICU	Troyes	+	-	-	-
18		NICU	Lyon	+	-	-	-
19		NICU	Lyon	+	-	-	-
20		NICU	Limoges	+	-	-	-
21		NICU	Limoges	+	-	-	-
22		NICU	Limoges	+	C	C	V
23		NICU	Limoges	+	C	C	V
24		NICU	Limoges	+	-	-	-
25		NICU	Limoges	+	-	-	-
26		NICU	Troyes	+	C	C	V
27		NICU	Lyon	+	-	-	-
28		NICU	Lyon	+	-	-	-
29		NICU	Lyon	+	C	C	V
30		NICU	Lyon	+	C	C	V
31		NICU	Lyon	+	-	-	-
32		NICU	Limoges	+	C	C	V
33		NICU	Lyon	+	-	-	-
34		NICU	Lyon	+	-	-	-
35		NICU	Lyon	+	-	-	-
36		NICU	Lyon	+	-	-	-
37		NICU	Lyon	+	C	C	V
38		NICU	Nantes	+	C	C	V
39		NICU	Caen	+	-	-	-
40		NICU	Caen	+	C	C	V
41	B	Adult Surgery	Lyon	-	-	-	-
42	C	NICU	Lyon	-	-	-	-
43	D	Adult ICU	Lyon	+	C	A2/B2/C	unkn.
44	E	Adult Medicine	Lyon	+	C	A2/B2/C	unkn.
45		Adult ICU	Lyon	+	-	-	-
46	F	Pediatric Medicine	Lyon	-	-	-	-
47	G	Adult Medicine	Versailles	+	C	C	V
48	H	Adult Medicine	Lyon	+	B	A2/B2	IV
49	I	Adult Medicine	Lyon	+	B	A2/B2	IV
50	J	Adult Medicine	Lyon	+	C	A2/B2/C	unkn.
51	K	Adult Medicine	Lyon	+	C	A2/B2/C	unkn.
52	L	Adult ICU	Lyon	+	B	A2/B2	IV
53	M	Adult Surgery	Lyon	-	-	-	-

Figure 2. *Staphylococcus capitis* isolates from distant neonatal intensive care units (NICUs) are clonal. Pulsed-field gel electrophoresis (PFGE) was applied to 53 bloodstream isolates of *S. capitis* that were collected from NICU infants and adult patients from cities spanning the French territory. The PFGE dendrogram was generated using the GelCompar software version 4.1. The isolates were assigned to pulsotypes using >80% similarity (vertical dashed line). Staphylococcal chromosomal cassette *mec* (SCC*mec*) typing was applied to 23 methicillin-resistant isolates representative of each pulsotype and geographic origin. All methicillin-resistant isolates from the different NICUs belonged to the same pulsotype and shared a type V-related SCC*mec* element, whereas methicillin-susceptible and/or non-NICU isolates were genetically diverse. *ccr*, chromosomal cassette recombinase; unkn., unknown (a combination of the *mec* complex and *ccr* genes has not been assigned to an SCC*mec* type so far).
doi:10.1371/journal.pone.0031548.g002

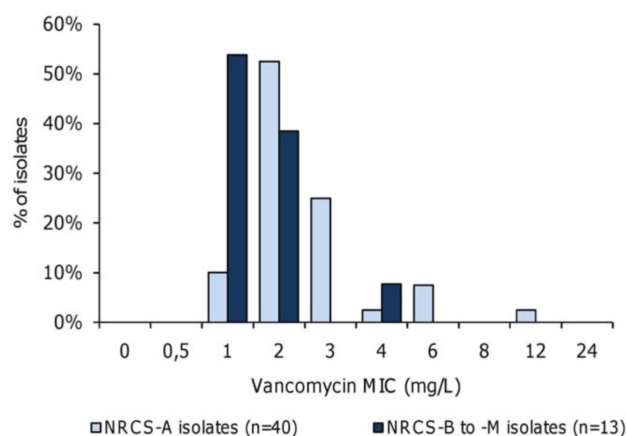

Figure 3. Vancomycin MICs are higher in *Staphylococcus capitis* pulsotype NRCS-A than in other pulsotypes. Vancomycin MICs were determined by the E-Test method. The mean vancomycin MIC was significantly higher in pulsotype NRCS-A isolates (2.8 mg/L) than in pulsotypes NRCS-B to –M isolates (1.7 mg/L), as illustrated by the shift toward the higher values of the MIC distribution curve ($P<0.01$, two-tailed Mann-Whitney *U*-test).
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Interestingly, previous reports of *S. capitis* in the NICU setting have shown strains of this species to be clustered in a single pulsotype [13,42]. It is unknown whether the spread of *S. capitis* NRCS-A is limited to France or if these strains are present in the NICUs of other countries. Molecular typing and a comparison of the isolates from international sources are warranted to address this question.

The reasons underlying the success of *S. capitis* pulsotype NRCS-A in the NICU environment are not completely understood. NICU isolates were found to be specifically resistant to the antimicrobial agents used in these wards, namely beta-lactams, aminosides and vancomycin [43], but not fluoroquinolones (Table 2). This adapted resistance profile likely results from a slow and progressive expansion of this clone in NICUs rather than from a rapid epidemic spread. Heteroresistance to vancomycin has been proposed as an intrinsic feature of the *S. capitis* species [23]. An increased tendency towards vancomycin resistance in pulsotype NRCS-A isolates may have been a decisive feature that led to their selection under high vancomycin pressure in the NICUs.

Whether *S. capitis* LOS in NICU infants is associated with higher morbidity or treatment failure compared to other CoNS pathogens is unknown. The present study was specifically designed to address the prevalence and molecular characteristics of *S. capitis* in the NICUs; therefore, we did not investigate the clinical course of *S. capitis* bacteremia. Our findings suggest the need for a prospective cohort study to determine whether specific risk factors are associated with LOS due to *S. capitis* and to what extent elevated vancomycin MICs in this pathogen correlate with an impaired response to glycopeptide therapy.

Our study has limitations in addition to those inherent to its retrospective design that need to be addressed. First, we used laboratory-based definitions of CoNS bacteremia in NICU infants, which determined that one CoNS-positive blood culture was considered to be bacteremic in this population. It is likely that a non-negligible proportion of CoNS-positive blood cultures included in the analysis were false-positive, thus introducing a bias towards an overestimation of CoNS prevalence as a cause of bacteremia. Nonetheless, it is unlikely that the large difference in *S. capitis* prevalence between NICU and adult ICU patients can be explained by this inclusion bias. Second, although we have shown that the *S. capitis* pulsotype NRCS-A is present in the NICUs of several French cities, our prevalence study was limited to the NICUs of a single town; therefore, it does not necessarily reflect the regional epidemiology of *S. capitis*. Third, the over-representation of isolates from Lyon in our collection introduces a potential underestimation of genetic diversity. However, because all of the NICU isolates collected outside of Lyon belonged to pulsotype NRCS-A, we can reasonably conclude that this sampling bias does not account for the observed clonality of *S. capitis* in the NICUs. Finally, one cannot exclude the possibility that this clonality resulted from a lack of discriminatory power of *Sma*I PFGE regarding the *S. capitis* species, but the high diversity of the PFGE banding patterns in the isolates from adult patients does not support this hypothesis. Furthermore, additional typing of a subset of NRCS-A isolates from different cities using repetitive sequence-based PCR [44] yielded indistinguishable banding patterns (data not shown) that confirmed the close genetic relationship between these isolates.

To conclude, the *S. capitis* pulsotype NRCS-A has adapted remarkably to the NICU setting; it has overtaken *S. epidermidis* as the leading pathogen that causes LOS in the NICUs of Lyon while remaining virtually absent from adult patients. Microbiologists and physicians in NICUs with high *S. capitis* prevalence should be aware of the potential reduced vancomycin susceptibility of this pathogen.

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Author Contributions

Conceived and designed the experiments: JPR OR ST FL. Performed the experiments: OR JPR MB CT. Analyzed the data: JPR OR FL. Contributed reagents/materials/analysis tools: ST JE. Wrote the paper: JPR FL JE JCP OC. Collected the retrospective data: ST JCP JG MBS OC.

References

1. Brodie SB, Sands KE, Gray JE, Parker RA, Goldmann DA, et al. (2000) Occurrence of nosocomial bloodstream infections in six neonatal intensive care units. Pediatr Infect Dis J 19: 56–65.
2. Stoll BJ, Gordon T, Korones SB, Shankaran S, Tyson JE, et al. (1996) Late-onset sepsis in very low birth weight neonates: a report from the National Institute of Child Health and Human Development Neonatal Research Network. J Pediatr 129: 63–71.
3. Gaynes RP, Edwards JR, Jarvis WR, Culver DH, Tolson JS, et al. (1996) Nosocomial infections among neonates in high-risk nurseries in the United States. National Nosocomial Infections Surveillance System. Pediatrics 98: 357–361.
4. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, et al. (2002) Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics 110: 285–291.
5. Gray JE, Richardson DK, McCormick MC, Goldmann DA (1995) Coagulase-negative staphylococcal bacteremia among very low birth weight infants: relation to admission illness severity, resource use, and outcome. Pediatrics 95: 225–230.
6. Freeman J, Goldmann DA, Smith NE, Sidebottom DG, Epstein MF, et al. (1990) Association of intravenous lipid emulsion and coagulase-negative staphylococcal bacteremia in neonatal intensive care units. N Engl J Med 323: 301–308.

7. Johnson-Robbins LA, el-Mohandes AE, Simmens SJ, Keiser JF (1996) *Staphylococcus epidermidis* sepsis in the intensive care nursery: a characterization of risk associations in infants <1,000 g. Biol Neonate 69: 249–256.
8. Freeman J, Epstein MF, Smith NE, Platt R, Sidebottom DG, et al. (1990) Extra hospital stay and antibiotic usage with nosocomial coagulase-negative staphylococcal bacteremia in two neonatal intensive care unit populations. Am J Dis Child 144: 324–329.
9. Kirchhoff LV, Sheagren JN (1985) Epidemiology and clinical significance of blood cultures positive for coagulase-negative *Staphylococcus*. Infect Control 6: 479–486.
10. Kim SD, McDonald LC, Jarvis WR, McAllister SK, Jerris R, et al. (2000) Determining the significance of coagulase-negative staphylococci isolated from blood cultures at a community hospital: a role for species and strain identification. Infect Control Hosp Epidemiol 21: 213–217.
11. Herwaldt LA, Geiss M, Kao C, Pfaller MA (1996) The positive predictive value of isolating coagulase-negative staphylococci from blood cultures. Clin Infect Dis 22: 14–20.
12. Klingenberg C, Ronnestad A, Anderson AS, Abrahamsen TG, Zorman J, et al. (2007) Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness. Clin Microbiol Infect 13: 1100–1111.
13. Bradford R, Abdul Manan R, Daley AJ, Pearce C, Ramalingam A, et al. (2006) Coagulase-negative staphylococci in very-low-birth-weight infants: inability of genetic markers to distinguish invasive strains from blood culture contaminants. Eur J Clin Microbiol Infect Dis 25: 283–290.
14. de Silva GD, Kantzanou M, Justice A, Massey RC, Wilkinson AR, et al. (2002) The *ica* operon and biofilm production in coagulase-negative staphylococci associated with carriage and disease in a neonatal intensive care unit. J Clin Microbiol 40: 382–388.
15. Klingenberg C, Sundsfjord A, Ronnestad A, Mikalsen J, Gaustad P, et al. (2004) Phenotypic and genotypic aminoglycoside resistance in blood culture isolates of coagulase-negative staphylococci from a single neonatal intensive care unit, 1989–2000. J Antimicrob Chemother 54: 889–896.
16. Ruhe J, Menon A, Mushatt D, Dejace P, Hasbun R (2004) Non-*epidermidis* coagulase-negative staphylococcal bacteremia: clinical predictors of true bacteremia. Eur J Clin Microbiol Infect Dis 23: 495–498.
17. Nalmas S, Bishburg E, Meurillio J, Khoobiar S, Cohen M (2008) *Staphylococcus capitis* prosthetic valve endocarditis: report of two rare cases and review of literature. Heart Lung 37: 380–384.
18. Cone LA, Sontz EM, Wilson JW, Mitruka SN (2005) *Staphylococcus capitis* endocarditis due to a transvenous endocardial pacemaker infection: case report and review of *Staphylococcus capitis* endocarditis. Int J Infect Dis 9: 335–339.
19. Ng PC, Chow VC, Lee CH, Ling JM, Wong HL, et al. (2006) Persistent *Staphylococcus capitis* septicemia in a preterm infant. Pediatr Infect Dis J 25: 652–654.
20. Van Der Zwet WC, Debets-Ossenkopp YJ, Reinders E, Kapi M, Savelkoul PH, et al. (2002) Nosocomial spread of a *Staphylococcus capitis* strain with heteroresistance to vancomycin in a neonatal intensive care unit. J Clin Microbiol 40: 2520–2525.
21. Wang SM, Liu CC, Tseng HW, Yang YJ, Lin CH, et al. (1999) *Staphylococcus capitis* bacteremia of very low birth weight premature infants at neonatal intensive care units: clinical significance and antimicrobial susceptibility. J Microbiol Immunol Infect 32: 26–32.
22. Gras-Le Guen C, Fournier S, Andre-Richet B, Caillon J, Chamoux C, et al. (2007) Almond oil implicated in a *Staphylococcus capitis* outbreak in a neonatal intensive care unit. J Perinatol 27: 713–717.
23. D'Mello D, Daley AJ, Rahman MS, Qu Y, Garland S, et al. (2008) Vancomycin heteroresistance in bloodstream isolates of *Staphylococcus capitis*. J Clin Microbiol 46: 3124–3126.
24. French Society for Microbiology (2009) Recommandations du Comite de l'Antibiogramme de la Societe Francaise de Microbiologie. Available at: wwwsfmassofr/doc/downloadphp?doc = DiU8C&fic = casfm_2009pdf Accessed 22 February 2009.
25. Hall KK, Lyman JA (2006) Updated review of blood culture contamination. Clin Microbiol Rev 19: 788–802.
26. European_Committee_on_Antimicrobial_Susceptibility_Testing (2010) Rationale for the EUCAST clinical breakpoints, version 2.1. Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Rationale_documents/Vancomycin_rationale_2.1.pdf. Accessed 5 October 2010.
27. Courcol R, Herrmann J, Laudat P, Pangon B, Peigue-Lafeuille H (2010) Conservation des souches, sérums et prélèvements biologiques. In: Référentiel en Microbiologie Médicale. Paris, France: Société Française de Microbiologie. pp 367.
28. Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, et al. (2001) Development of a PCR assay for identification of staphylococci at genus and species levels. J Clin Microbiol 39: 2541–2547.
29. Goering RV (1993) Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. Infect Control Hosp Epidemiol 14: 595–600.
30. George CG, Kloos WE (1994) Comparison of the *Sma*I-digested chromosomes of *Staphylococcus epidermidis* and the closely related species *Staphylococcus capitis* and *Staphylococcus caprae*. Int J Syst Bacteriol 44: 404–409.
31. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, et al. (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41: 5113–5120.
32. Grundmann H, Hori S, Tanner G (2001) Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. J Clin Microbiol 39: 4190–4192.
33. Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, et al. (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob Agents Chemother 51: 264–274.
34. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (2009) Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother 53: 4961–4967.
35. Vaudaux P, Huggler E, Bernard L, Ferry T, Renzoni A, et al. (2010) Underestimation of vancomycin and teicoplanin MICs by broth microdilution leads to underdetection of glycopeptide-intermediate isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother 54: 3861–3870.
36. Nadarajah R, Post LR, Liu C, Miller SA, Sahm DF, et al. (2010) Detection of vancomycin-intermediate *Staphylococcus aureus* with the updated Trek-Sensititre System and the MicroScan System. Comparison with results from the conventional Etest and CLSI standardized MIC methods. Am J Clin Pathol 133: 844–848.
37. Satola SW, Farley MM, Anderson KF, Patel JB (2011) Comparison of detection methods for heteroresistant vancomycin-intermediate *Staphylococcus aureus*, with the population analysis profile method as the reference method. J Clin Microbiol 49: 177–183.
38. Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, et al. (1997) Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350: 1670–1673.
39. European Committee on Antimicrobial Susceptibility Testing (2010) Clinical breakpoints. Available at: http://www.eucast.org/clinical_breakpoints. Accessed 5 October 2010.
40. Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales (Raisin) (2009) Enquête nationale de prévalence des infections nosocomiales, France, juin 2006. Saint-Maurice, France: Institut de Veille Sanitaire. Available at: http://www.invs.sante.fr/surveillance/raisin/. Accessed 5 October 2011.
41. Centre de Coordination de la Lutte contre les Infections Associées aux Soins (2011) Rapport de la surveillance 2010 du réseau NEOCAT. Available at: http://www.cclinparisnord.org/NEOCAT/2010/NEOCAT10_Rapport.pdf. Accessed 15 October 2011.
42. de Silva GD, Justice A, Wilkinson AR, Buttery J, Herbert M, et al. (2001) Genetic population structure of coagulase-negative staphylococci associated with carriage and disease in preterm infants. Clin Infect Dis 33: 1520–1528.
43. Patel SJ, Oshodi A, Prasad P, Delamora P, Larson E, et al. (2009) Antibiotic use in neonatal intensive care units and adherence with Centers for Disease Control and Prevention 12 Step Campaign to Prevent Antimicrobial Resistance. Pediatr Infect Dis J 28: 1047–1051.
44. Grisold AJ, Zarfel G, Strenger V, Feierl G, Leitner E, et al. (2010) Use of automated repetitive-sequence-based PCR for rapid laboratory confirmation of nosocomial outbreaks. J Infect 60: 44–51.

ANNEXE VII

Rise of CC398 Lineage of *Staphylococcus aureus* among Infective Endocarditis Isolates Revealed by Two Consecutive Population-Based Studies in France

Tristan A, Rasigade JP, Ruizendaal E, Laurent F, Bes M, Meugnier H, Lina G, Etienne J, Celard M, Tattevin P, Monecke S, Le Moing V, Vandenesch F and the French AEPEI Study Groupe on Infective Endocarditis

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Rise of CC398 Lineage of *Staphylococcus aureus* among Infective Endocarditis Isolates Revealed by Two Consecutive Population-Based Studies in France

Anne Tristan[1,2,3]*, Jean-Philippe Rasigade[1,2,3], Esmée Ruizendaal[1], Frédéric Laurent[1,2,3], Michèle Bes[1,2,3], Hélène Meugnier[1], Gérard Lina[1,2,3], Jerome Etienne[1,2,3], Marie Celard[1], Pierre Tattevin[4], Stefan Monecke[5], Vincent Le Moing[6], François Vandenesch[1,2,3], the French AEPEI study Group on Infective Endocarditis

1 Hospices Civils de Lyon, Centre National de Référence des Staphylocoques, Bron, France, **2** Université de Lyon, Domaine de la Buire, Lyon, France, **3** INSERM U851, Bacterial Pathogenesis and Innate Immunity Laboratory, Lyon, France, **4** Infectious Diseases and Intensive Care Unit, Pontchaillou University Hospital, Rennes, France, **5** Institute for Medical Microbiology and Hygiene, Faculty of Medicine "Carl Gustav Carus", Technical University of Dresden, Dresden, Germany, **6** Département des maladies infectieuses et tropicales, hôpital Gui-de-Chauliac, Montpellier, France

Abstract

Staphylococcus aureus isolates from two prospective studies on infective endocarditis (IE) conducted in 1999 and 2008 and isolated from non-IE bacteremia collected in 2006 were *spa*-typed and their virulence factors were analyzed with a microarray. Both populations were genetically diverse, with no virulence factors or genotypes significantly more associated with the IE isolates compared with the non-IE isolates. The population structure of the IE isolates did not change much between 1999 and 2008, with the exception of the appearance of CC398 methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates responsible for 5.6% of all cases in 2008. In 1999, this lineage was responsible for no cases. The increasing prevalence of *S. aureus* in IE is apparently not the result of a major change in staphylococcal population structure over time, with the exception of the emerging CC398 MSSA lineage.

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* E-mail: anne.tristan@univ-lyon1.fr

Introduction

Infective endocarditis (IE) is a rare but severe disease, which characteristics have changed over the past decades, and a shift in causative microorganisms has been observed. Staphylococci have surpassed streptococci as the primary group of IE-causing pathogens [1], as confirmed by a number of observational studies [2,3]. Our study group conducted three population-based studies of IE in 1991, 1999 and 2008, using the same methods, based on a quarter of the French population [4–6]. These studies revealed an increasing *S. aureus* prevalence over the two decades covered by those three individual year time points (16.1%, 21.1%, and 25.7% for 1991, 1999 and 2008, respectively) [4]. In the 2008 survey, although the streptococci as a group were more frequently observed, *S. aureus* was the leading single-species cause of IE. Moreover, healthcare-associated IE accounted for almost 25% of all IE cases [6]. This result is consistent with other studies that previously indicated a shift from IE, mostly of dental origin, to a predominance of healthcare-related infections [7]. These evolutions in the microbial epidemiology of IE have been attributed to population aging, changes in predisposing cardiac conditions, and bloodstream infection (BSI) patterns [8]. However, no study has examined whether a qualitative change of the microorganisms responsible for IE, such as a shift in the causative lineages or the emergence of new clones of *S. aureus*, has occurred over time.

Another important question concerns the specificity of infective endocarditis strains with respect to other *S. aureus* infections. When comparing methicillin-susceptible *S. aureus* (MSSA) from uncomplicated infections (uncomplicated bacteremia and soft tissue infections) with those isolated from IE or haematogenous bone and joint infections within the same hospital, Fowler *et al* found that CC5 and CC9 were associated with an increased severity of infection [9]. In another study comparing geographically matched complicated skin- and soft-tissue infection (SSTI) MSSA isolates with IE MSSA isolates, Nienaber *et al* found that CC30 was the sole genotype that was significantly more frequent in IE than in SSTI isolates [10]. However, the question remains as to whether *S. aureus* IE isolates differ from non-IE bacteremia isolates. Finally, one striking observation by Nienaber *et al* [10] is that the genes encoding toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin A (SEA), two major superantigens from *S. aureus*, were extremely prevalent in IE isolates from the United States (93.9% and 64.9% respectively). These results suggest that IE isolates harbor specific virulence factors that differ from those found in isolates sampled from people suffering other diseases.

In the present paper, we thoroughly analyzed a collection of *S. aureus* IE isolates collected in 2008 during a population-based survey that covered a quarter of French territory. To assess the temporal changes associated with IE-causing staphylococci, we compared the population structure of these isolates with those from a similar survey conducted in 1999. To determine the specificity of IE isolates versus non-IE bacteremia isolates we compared the population structure and the virulence gene content of IE isolates from the 2008 survey with those from non-IE bacteremia isolates collected throughout France in 2006–2007.

Results

Genotyping of IE MSSA isolates

The genotypes of the 89 MSSA IE isolates were analyzed both by *spa* typing and by the use of a microarray. The microarray assigned all isolates to clonal complexes (CCs). Overall, there was a large genotypic diversity as exemplified by the 18 different CCs detected. However, four CCs (CC45, CC5, CC15 and CC30) accounted for >50% of the 89 isolates (Table 1). Among the less frequent CCs, five isolates (5.6%) belonged to CC398. This assignment was verified by a CC398-specific PCR [11]. The genetic diversity of the IE isolates was further confirmed by *spa*-typing indicating that the 89 isolates were distributed among 63 *spa* types and 11 *spa* clusters, with 5 *spa* clusters accounting for >50% of the isolates.

To determine whether the population structure of the IE isolates was stable over time, the *spa* types of the *S. aureus* isolates collected during the 1999 French IE survey were determined. The results indicated that the two groups of isolates were genotypically very comparable with the *spa* types of the 2008 isolates interspaced with those of 1999 isolates (Figure 1). The exception was CC398, as none of the *spa* cluster identified in 1999 could be assigned to CC398.

Correlation between CC and disease parameters

In order to determine whether certain lineages of *S. aureus* could be associated with certain clinical characteristics of the patients, search of associations between CC (assigned by the microarrays) and the patient database of the 2008 survey [6] was performed. The only significant association detected was between intravenous drug addiction and CC8. Four of the 8 CC8 isolates (50.0%) versus 14 of the 81 non-CC8 isolates (17.3%) were associated with IV drug abuse ($P<0.05$, two-tailed Fisher's exact test).

Comparison of IE to non-IE blood-culture MSSA isolates

To assess whether IE isolates correspond to peculiar lineages and harbor specific virulence factors in comparison to non-IE isolates, the population structure of the 89 MSSA IE isolates of the 2008 survey was compared with that of the 81 blood culture non-IE isolates collected in France during 2006. Genotypic comparison, as assessed both by *spa* typing and microarrays, revealed that the two populations were genotypically superimposable in regard to CCs (inferred from microarrays) (Table 1) and *spa* clusters (Figure 2). Figure 2 depicts that each *spa* cluster contained both IE and non-IE isolates. The two isolate populations were then compared to determine the prevalence of individual virulence factor genes. Genomic DNA microarray analysis comparison of the 89 MSSA IE isolates with the 81 non-IE bacteremia isolates indicated that there were no specific genes (including virulence factor genes) that could be significantly assigned to IE versus bacteremia isolates (Table 2 and Table S1). The known adhesin genes present in at least 60% of isolates of both groups were *fnb*AB (fibronectin binding protein A–B), *clf*AB (clumping factor A–B), *spa* (protein A), *sdr*CD (ser-asp rich fibrinogen-binding, bone sialoprotein-binding protein C and D), *bbp* (bone sialoprotein-binding protein), *ebp*S (cell wall associated fibronectin-binding protein) and *map* (major histocompatibility complex class II analog protein). Similarly, the exotoxins and other putative virulence factors present in at least 60% of isolates in both groups were *seg/sei* (encoding the *egc* cluster), *ica*A (intercellular adhesion protein) and *chp* (chemotaxis inhibitory protein) (Table 2).

Discussion

A wide range of different *S. aureus* clonal complexes were found to be involved in IE. Altogether, 18 different CCs were detected. This distribution did not vary much over time as assessed by the *spa*-typing comparison of the 1999 and 2008 isolates (Figure 1). This observation suggests that the increased prevalence of *S. aureus* accompanying endocarditis is not due to a change in bacterial population structure but rather mainly due to non-bacterial factors, including population aging and an increase in conditions favoring *S. aureus* endocarditis, such as the use of prosthetic valves, pace-makers and hospitalization [6]. An exception to the general stability of the *S. aureus* population structure over time is the identification of five CC398 strains in the 2008 survey. CC398 includes MSSA and methicillin-resistant *S. aureus* (MRSA), the latter being associated with livestock-associated infections, mainly in pigs [12], although cases of infections in humans with this

Table 1. A comparison of the population structures of methicillin-susceptible *Staphylococcus aureus* isolates from patients with infective endocarditis (IE) or bloodstream infection (BSI) without IE[a].

MLST Clonal complex (CC)[b]	IE isolates (%), n = 89	non-IE BSI isolates (%), n = 81	P-value[c]
CC45	16 (18.0)	14 (17.3)	1.000
CC5	16 (18.0)	15 (18.5)	1.000
CC15	11 (12.4)	4 (4.9)	0.108
CC30	11 (12.4)	11 (13.6)	0.823
CC8	8 (9.0)	10 (12.3)	0.619
CC398	5 (5.6)	2 (2.5)	0.447
Others	22 (24.7)	25 (30.9)	0.395

[a]IE and non-IE isolates were collected in 2008 and 2006, respectively.
[b]MLST Clonal Complexes were inferred from microarray analysis.
[c]P-values were calculated for each CC using a two-tailed Fisher's exact test. The P-value for the whole contingency table was 0.592.
doi:10.1371/journal.pone.0051172.t001

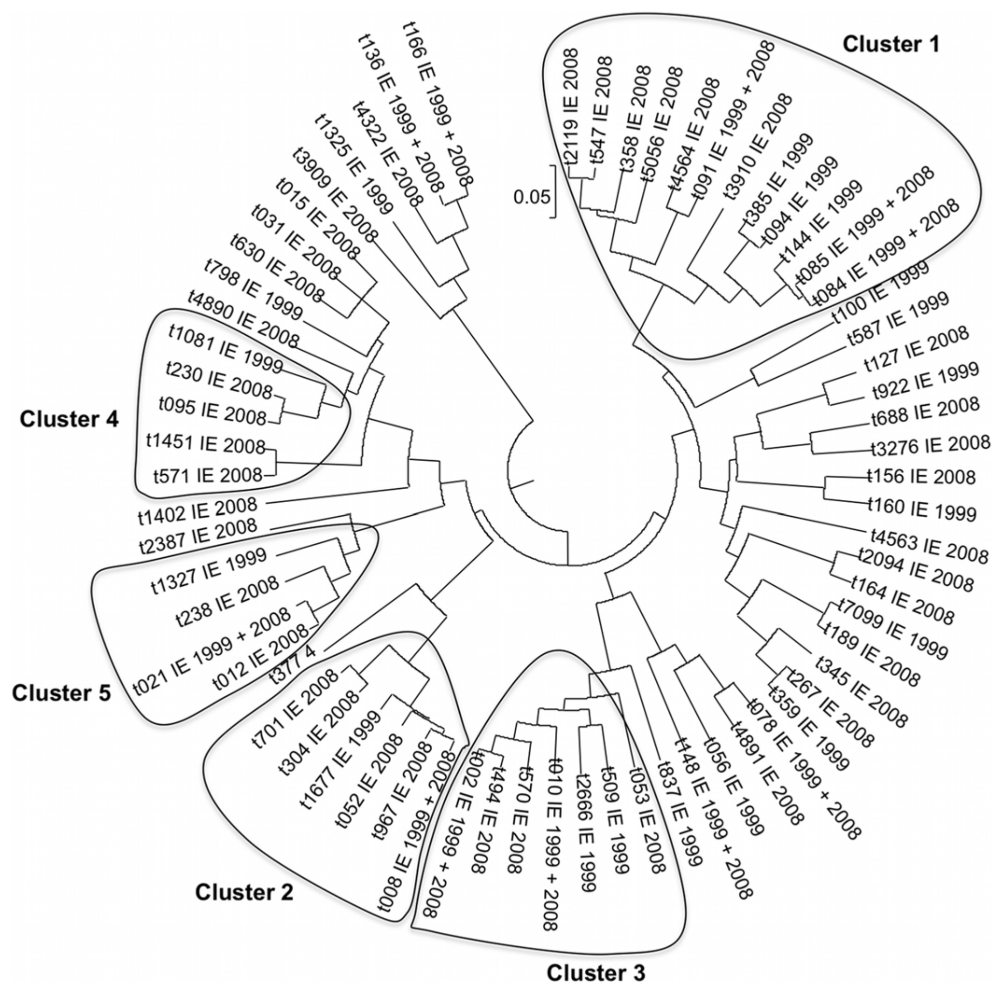

Figure 1. Phylogenetic tree based on *spa* type of *S. aureus* IE isolates from 1999 and 2008 surveys. Figure 1. Circular representation of the phylogenetic tree obtained using UPGMA method (http://spa.ridom.de/). The *spa* types were clustered into CCs (ie, *spa*CCs) by use of the integrated BURP (Based Upon Repeat Patterns) algorithm. User-definable parameters were set as follows: ''cluster *spa* types into *spa*CC if cost distances are less than or equal to 4'' and ''exclude *spa* types shorter than 5 repeats.'' This parameter combination ensures optimal concordance (95.3%) between BURP and e- BURST (http://spa.ridom.de/). Only major *spa*CC are represented.
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lineage have been described [13]. Only limited documentation of CC398 MSSA strains exists [14]. However, with a frequency of 5 out of 89 isolates (5.6%), CC398 MSSA cannot be neglected as an important IE pathogen in humans. Other reports suggest that CC398 is a new emerging pathogenic lineage in humans [13,15–17]. Future studies are needed to determine whether this lineage will continue to expand throughout the population.

When comparing IE versus non-IE isolates from the same geographic area, we found no difference in the population structure between the IE and non-IE bacteremia isolates. CC5 and CC45 dominate in both populations and CC398 was equally prevalent in IE and non-IE isolates (Table 1). This result is consistent with other studies, which also found highly heterogenic strains in *S. aureus* infection without any particular CC dominating involvement in invasive infections [18]. Of note, CC30 was slightly less represented in the two populations (13.6% in IE, 12.4% in non-IE infection) than it was in the Nienaber study (19.5% in IE) [10].

The population structure of our IE and non-IE isolates does not appear to be different from that observed with other invasive or colonizing isolate series. For instance, the population structure of MSSA from 943 invasive infections in the Netherland over a period of 11 years revealed that a majority of isolates belonged to the same CCs as our series [18]. Interestingly, nasal carriage isolates from a collection of 155 carriers (152 MSSA and 3 MRSA) sampled in Germany belonged, in a decreasing order of prevalence, to CC8, CC30, CC15 and CC45, which correspond to four of the five most prevalent CCs in our series [19]. Likewise, when comparing CC affiliations of IE isolates to *S. aureus* isolate typing data obtained from healthy carriers or bone infections in Germany [19,20], a similar pattern can be observed. In line with the lack of correlation between CC and diseases vs carriage, we found no major association between CC and clinical characteristics of the infective endocarditis with the exception of CC8 with IV drug abuse. Although significant, this association relies on a small population. In the context of IV drug abuse, this association may reflect a propensity of the CC8 lineage to better colonize the skin.

When considering the virulence factors potentially associated with IE, a number of staphylococcal adhesins are considered to

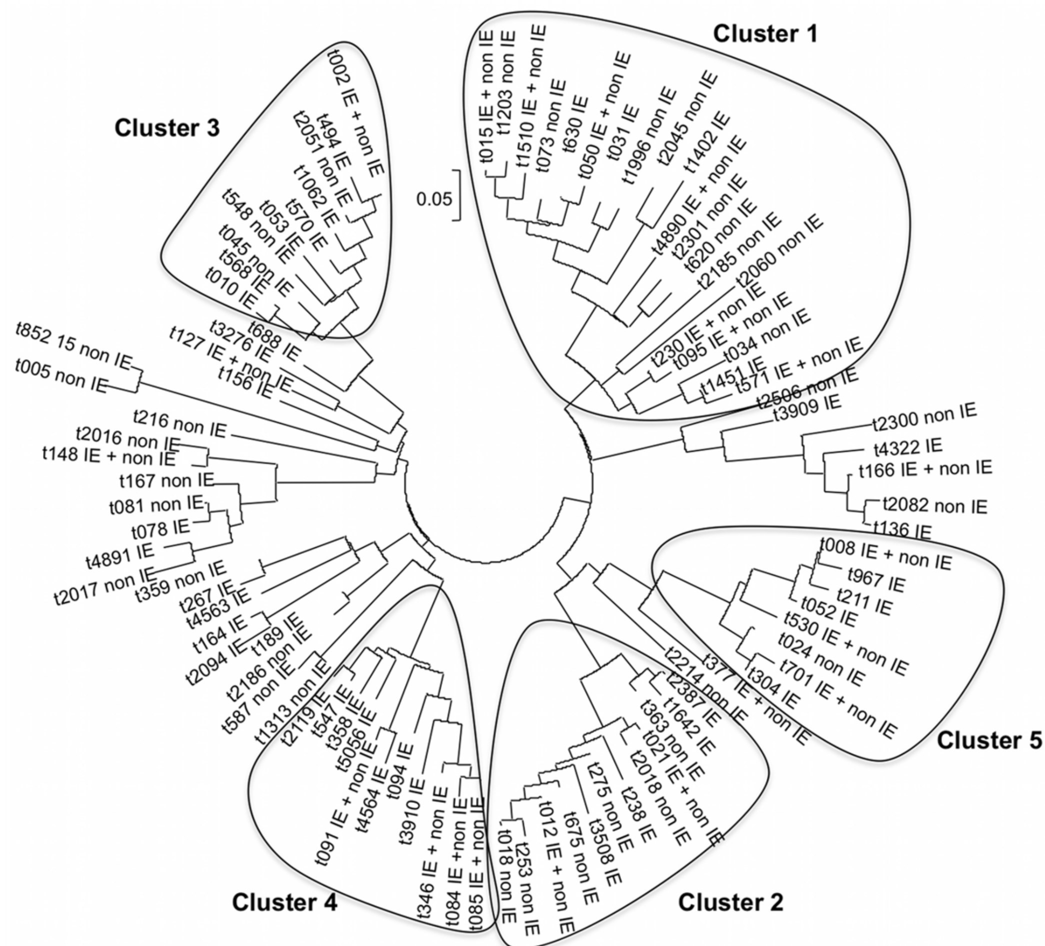

Figure 2. Phylogenetic tree based on *spa* type of *S. aureus* IE isolates (2008 survey) and bloodstream infections without IE (2006 survey). See Figure 1 legend for details.
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play a role in the pathogenesis of infective endocarditis, both *in vitro* or when using animal experimental models. This list includes clumping factor A–B [21,22], fibronectin-binding protein A–B [21], collagen-binding protein [23], SdrD/E [22] and polysaccharide intercellular adhesin [24]. In our series of IE isolates, these factors, with the exception of collagen-binding protein gene (*cna*), were highly prevalent; however, they were equally prevalent in non-IE bacteremia isolates (Table 2), suggesting that they might be required but are not sufficient for the development of IE. All other variable virulence genes were equally prevalent in the two populations (Table S1). Altogether, it appears that the population structure and prevalence of variable virulence genes are not significantly different between invasive isolates responsible for bloodstream infections. These observations do not rule out the possibility of differential expression of virulence factors between isolates as suggested by some studies [25].

As mentioned in the introduction, TSST-1 and SEA-encoding gene were extremely prevalent in IE isolates from the United States (93.9% and 64.9% respectively) [10]. These results are quite different from our epidemiology for both our IE isolates (9% *tst* and 18% *sea*) and non-IE bacteremia isolates (19.8% *tst* and 21% *sea*), as well as different from the collection of nasal carriage studied by Monecke *et al* with the same arrays (14.84% *tst* and 17.42% *sea*) [19]. As mentioned by Nienaber, the high prevalence of major superantigens in their series could "reflect linkage disequilibrium with unidentified virulence genes and represent a 'biomarker' of *S. aureus* isolates with an increased risk for IE rather than a causal association" [10]. Whole-genome sequencing of these isolates is currently under way (V. Fowler, personal communication) and may address this important issue. Finally, Panton-Valentine leukocidin (PVL) was not detected in any IE or bloodstream infection isolates from our series and was detected at a frequency below 20% in the US series [10].

There is one limitation to the present study. The strains collected from the non-IE patients were retrieved from a survey in which IE was not formerly ruled out by performing a transesophageal echocardiography (TEE) [26]. Thus, a few cases of IE may have been misdiagnosed in the present non-IE bacteremia series. However, given the expected prevalence of IE within the bacteremia isolates from various studies (5–10% in healthcare-related, 10 to 15% in community-acquired *S. aureus* bacteremia) [27,28], this bias is unlikely to have had a significant impact on our observation that IE and non-IE isolates carry similar genetic backgrounds and virulence profile genes. However, a current

Table 2. A comparison of the genotypic profiles of methicillin-susceptible *Staphylococcus aureus* isolates from patients with infective endocarditis (IE) or bloodstream infection (BSI) without IE[a].

Gene or allele	IE isolates (%), n = 89	non-IE BSI isolates (%), n = 81	P-value[b]
Adhesins			
fnbA	89 (100.0)	79 (97.5)	0.226
fnbB	76 (85.4)	70 (86.4)	1.000
clfA	89 (100.0)	81 (100.0)	1.000
clfB	89 (100.0)	81 (100.0)	1.000
cna	37 (41.6)	33 (40.7)	1.000
spa	89 (100.0)	81 (100.0)	1.000
sdrC	89 (100.0)	81 (100.0)	1.000
sdrD	73 (82.0)	62 (76.5)	0.449
bbp	78 (87.6)	78 (96.3)	0.051
ebpS	89 (100.0)	81 (100.0)	1.000
map/eap	85 (95.5)	80 (98.8)	0.370
Toxins			
eta	0 (0.0)	1 (1.2)	0.476
etb	0 (0.0)	0 (0.0)	1.000
tst	8 (9.0)	16 (19.8)	0.050
sea	16 (18.0)	17 (21.0)	0.699
seb	1 (1.1)	5 (6.2)	0.104
sec	16 (18.0)	12 (14.8)	0.680
sed	5 (5.6)	4 (4.9)	1.000
see	0 (0.0)	0 (0.0)	1.000
seg	52 (58.4)	55 (67.9)	0.209
seh	6 (6.7)	6 (7.4)	1.000
sei	51 (57.3)	56 (69.1)	0.116
sej	5 (5.6)	4 (4.9)	1.000
pvl	0 (0.0)	0 (0.0)	1.000
hla	84 (94.4)	81 (100.0)	0.060
Other putative virulence genes			
icaA	89 (100.0)	81 (100.0)	1.000
chp	56 (62.9)	53 (65.4)	0.751
***agr* alleles**			
agr I	44 (49.4)	42 (51.9)	0.761
agr II	30 (33.7)	22 (27.2)	0.406
agr III	14 (15.7)	17 (21.0)	0.429
agr IV	1 (1.1)	0 (0.0)	1.000

[a]IE and non-IE isolates were collected in 2008 and 2006, respectively.
[b]P-values were calculated for each gene or allele using a two-tailed Fisher's exact test.
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prospective study matching IE and non-IE cases, both ascertained by the performance of a TEE, will provide a definite answer to this important question.

In conclusion, our data reveal that there was a wide genetic diversity within MSSA strains, with no virulence factors or CC particularly more involved in IE as compared to non-IE bacteremia, suggesting that the occurrence of infective endocarditis during *S. aureus* bacteremia depends essentially on host factors. The population structure of IE isolates changed little between the two surveys performed 9 years apart with the exception of an emerging phenomenon, the rise of the CC398 MSSA lineage as a cause of invasive infections.

Materials and Methods

Bacterial strains

IE Isolates. A collection of 89 MSSA, and 39 MSSA IE non-duplicate isolates collected during respective 2008 [6] and 1999 [5] population-based surveys was included in the study. These isolates were from confirmed cases of MSSA IE as determined by the modified Duke criteria [29]. Both one-year surveys (1999 and 2008) were conducted using the same methods. However, the 2008 study covered a larger area of French territory than the 1999 survey. Hence, when comparing the isolates of the two studies, the 2008 collection was restricted to the 78 MSSA isolates collected in the same area as the 1999 survey.

Non-IE isolates. Eighty-one blood culture isolates out of 116 invasive infection isolates (all MSSA) collected during a prospective multicenter study from September 2006 to February 2007 by 23 representative French hospital laboratories were included [26].

Genotyping methods

Bacterial DNA was extracted according to the manufacturer's recommended protocol using commercial extraction kits (Qiagen). The diagnostic DNA microarrays, Identibac *S. aureus* Genotyping ® (Alere) used for this study, as well as related procedures and protocols, have been previously described in detail [19]. This microarray covers 332 different target sequences corresponding to approximately 185 distinct genes and their allelic variants. The assignation of isolates to clonal complexes (CCs) was determined by the comparison of hybridization profiles to previously typed multilocus sequence typing (MLST) reference strains [19].

A DNA sequence-based analysis of the protein A gene variable region was performed as previously described [30] using the nomenclature as described on the Ridom website (http://spa.ridom.de/). To infer clonal relatedness, the Based Upon Repeat Pattern (BURP) algorithm was used followed by circular representation of the phylogenetic tree obtained using UPGMA method (http://spa.ridom.de/) [31].

Clinical characteristics of patient

The patient information of the 2008 survey had been collected prospectively by use of a standardized case report form as described [6]. The following variable were collected: sex, date of birth, living place, date of first symptoms and of first hospitalization, transfer from/to another facility, history of heart disease, comorbidities, Charlson index, procedures and situations at risk for IE, signs and symptoms of IE, echocardiographic data, microbiological data, laboratory and imaging investigations, medical and surgical treatment, and outcome [6].

Statistical analysis

Differences in the distributions of genes, alleles, genotypes and clinical features were tested for significance using a two-tailed Fisher's exact test. Contingency tables larger than 2×2 used in population structure comparisons were first analyzed as a whole, then each proportion was individually tested for significance. To reflect the exploratory nature of the analysis, P-values were not corrected for multiple testing. P-values of <0.05 were considered to be statistically significant. Statistical analyses were performed using R software version 2.14.2 (The R Foundation for Statistical Computing, Vienna, Austria).

Supporting Information

Table S1 A comparison of the distributions of genes or alleles in methicillin-susceptible *Staphylococcus aureus isolates* from patients with infective endocarditis (IE) or bloodstream infection (BSI) without IE (only genes not shown in Table1 are presented).
(DOCX)

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AEPEI Study Group on Infective Endocarditis

Principal investigators: B. Hoen, X. Duval; Other members: F. Alla, A. Bouvet, S. Briançon, N. Danchin, F. Delahaye, X. Duval, J. Etienne, V. Le Moing, C. Leport, C. Selton-Suty, and F. Vandenesch

Author Contributions

Conceived and designed the experiments: AT FL MB PT VLM FV. Performed the experiments: AT MB HM SM. Analyzed the data: AT ER JPR FL MB GL JE MC. Contributed reagents/materials/analysis tools: SM. Wrote the paper: AT ER JPR MB PT VLM FV.

References

1. Moreillon P, Que YA (2004) Infective endocarditis. Lancet 363: 139–149.
2. Fowler VGJ, Miro JM, Hoen B, Cabell CH, Abrutyn E, et al. (2005) *Staphylococcus aureus* endocarditis: a consequence of medical progress. JAMA 293: 3012–3021.
3. Murdoch DR, Corey GR, Hoen B, Miro JM, Fowler VGJ, et al. (2009) Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis-Prospective Cohort Study. Arch Intern Med 169: 463–473.
4. Duval X, Delahaye F, Alla F, Tattevin P, Obadia JF, et al. (2012) Temporal trends in infective endocarditis in the context of prophylaxis guideline modifications: three successive population-based surveys. J Am Coll Cardiol 59: 1968–1976.
5. Hoen B, Alla F, Selton-Suty C, Beguinot I, Bouvet A, et al. (2002) Changing profile of infective endocarditis: results of a 1-year survey in France. JAMA 288: 75–81.
6. Selton-Suty C, Celard M, Le Moing V, Doco-Lecompte T, Chirouze C, et al. (2012) Preeminence of *Staphylococcus aureus* in infective endocarditis: a 1-year population-based survey. Clin Infect Dis 54: 1230–1239.
7. Thuny F, Avierinos JF, Habib G (2010) Changing patterns in epidemiological profiles and prevention strategies in infective endocarditis: from teeth to healthcare-related infection. Eur Heart J 31: 1826–1827.
8. Cabell CH, Jollis JG, Peterson GE, Corey GR, Anderson DJ, et al. (2002) Changing patient characteristics and the effect on mortality in endocarditis. Arch Intern Med 162: 90–94.
9. Fowler VGJ, Nelson CL, McIntyre LM, Kreiswirth BN, Monk A, et al. (2007) Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. J Infect Dis 196: 738–747.
10. Nienaber JJ, Sharma Kuinkel BK, Clarke-Pearson M, Lamlertthon S, Park L, et al. (2011) Methicillin-Susceptible *Staphylococcus aureus* Endocarditis Isolates Are Associated With Clonal Complex 30 Genotype and a Distinct Repertoire of Enterotoxins and Adhesins. J Infect Dis 204: 704–713.
11. Stegger M, Lindsay JA, Moodley A, Skov R, Broens EM, et al. (2011) Rapid PCR detection of *Staphylococcus aureus* clonal complex 398 by targeting the restriction-modification system carrying sau1-hsdS1. J Clin Microbiol 49: 732–734.
12. Armand-Lefevre L, Ruimy R, Andremont A (2005) Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg Infect Dis 11: 711–714.
13. van Belkum A, Melles DC, Peeters JK, van Leeuwen WB, van Duijkeren E, et al. (2008) Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg Infect Dis 14: 479–483.
14. Mediavilla JR, Chen L, Uhlemann AC, Hanson BM, Rosenthal M, et al. (2012) Methicillin-Susceptible *Staphylococcus aureus* ST398, New York and New Jersey, USA. Emerg Infect Dis 18(4): 700–702.
15. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, et al. (2012) *Staphylococcus aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. MBio 3(1).
16. Uhlemann AC, Porcella SF, Trivedi S, Sullivan SB, Hafer C, et al. (2012) Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with distinct genomic and cell adhesion properties. MBio 3(2).
17. Valentin-Domelier AS, Girard M, Bertrand X, Violette J, Francois P, et al. (2011) Methicillin-Susceptible ST398 *Staphylococcus aureus* Responsible for Bloodstream Infections: An Emerging Human-Adapted Subclone? PLoS One 6: e28369.
18. Rijnders MI, Deurenberg RH, Boumans ML, Hoogkamp-Korstanje JA, Beisser PS, et al. (2009) Population structure of *Staphylococcus aureus* strains isolated from intensive care unit patients in the netherlands over an 11-year period (1996 to 2006). J Clin Microbiol 47: 4090–4095.
19. Monecke S, Luedicke C, Slickers P, Ehricht R (2009) Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. Eur J Clin Microbiol Infect Dis 28: 1159–1165.
20. Luedicke C, Slickers P, Ehricht R, Monecke S (2010) Molecular fingerprinting of *Staphylococcus aureus* from bone and joint infections. Eur J Clin Microbiol Infect Dis 29: 457–463.
21. Entenza JM, Moreillon P, Senn MM, Kormanec J, Dunman PM, et al. (2005) Role of sigmaB in the expression of *Staphylococcus aureus* cell wall adhesins ClfA

and FnbA and contribution to infectivity in a rat model of experimental endocarditis. Infect Immun 73: 990–998.
22. O'Brien L, Kerrigan SW, Kaw G, Hogan M, Penades J, et al. (2002) Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. Mol Microbiol 44: 1033–1044.
23. Hienz SA, Schennings T, Heimdahl A, Flock JI (1996) Collagen binding of *Staphylococcus aureus* is a virulence factor in experimental endocarditis. J Infect Dis 174: 83–88.
24. Zhu Y, Xiong YQ, Sadykov MR, Fey PD, Lei MG, et al. (2009) Tricarboxylic acid cycle-dependent attenuation of *Staphylococcus aureus* in vivo virulence by selective inhibition of amino acid transport. Infect Immun 77: 4256–4264.
25. Melles DC, Gorkink RF, Boelens HA, Snijders SV, Peeters JK, et al. (2004) Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J Clin Invest 114: 1732–1740.
26. Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, et al. (2010) Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med 7: e1000215.
27. Chang FY, MacDonald BB, Peacock JEJ, Musher DM, Triplett P, et al. (2003) A prospective multicenter study of *Staphylococcus aureus* bacteremia: incidence of endocarditis, risk factors for mortality, and clinical impact of methicillin resistance. Medicine (Baltimore) 82: 322–332.
28. Kaasch AJ, Fowler VGJ, Rieg S, Peyerl-Hoffmann G, Birkholz H, et al. (2011) Use of a simple criteria set for guiding echocardiography in nosocomial *Staphylococcus aureus* bacteremia. Clin Infect Dis 53: 1–9.
29. Li JS, Sexton DJ, Mick N, Nettles R, Fowler VGJ, et al. (2000) Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis 30: 633–638.
30. Harmsen D, Claus H, Witte W, Rothganger J, Claus H, et al. (2003) Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol 41: 5442–5448.
31. Mellmann A, Weniger T, Berssenbrugge C, Rothganger J, Sammeth M, et al. (2007) Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on spa polymorphisms. BMC Microbiol 7: 98.

Table S1. A comparison of the distributions of genes or alleles in methicillin-susceptible *Staphylococcus aureus isolates* from patients with infective endocarditis (IE) or bloodstream infection (BSI) without IE[a].

Gene or allele[b]	IE isolates (%), n=89	Non-IE BSI isolates (%), n=81	P-value[c]
SPECIES MARKER			
*rrn*D1 (domaine 1 of 23S-rRNA)	89 (100.0)	81 (100.0)	1.000
*gap*A	88 (98.9)	81 (100.0)	1.000
*kat*A	87 (97.8)	81 (100.0)	0.498
*Co*A	88 (98.9)	80 (98.8)	1.000
*nuc*1	87 (97.8)	81 (100.0)	0.498
sbi	86 (96.6)	81 (100.0)	0.247
REGULATORY GENES			
*sar*A	89 (100.0)	81 (100.0)	1.000
*sae*S	89 (100.0)	81 (100.0)	1.000
*vra*S	89 (100.0)	80 (98.8)	0.476
hld	88 (98.9)	81 (100.0)	1.000
RESISTANCE : PENICILLINASE			
*bla*Z	75 (84.3)	58 (71.6)	0.062
RESISTANCE : MLS-ANTIBIOTICS			
*erm*A	1 (1.1)	6 (7.4)	0.055
*erm*B	0 (0.0)	1 (1.2)	0.476
*erm*C	4 (4.5)	3 (3.7)	1.000
*lin*A	0 (0.0)	0 (0.0)	1.000
*msr*A	3 (3.4)	2 (2.5)	1.000
*mef*A	0 (0.0)	0 (0.0)	1.000
*mpb*BM	1 (1.1)	0 (0.0)	1.000
vatA	1 (1.1)	0 (0.0)	1.000
vatB	0 (0.0)	0 (0.0)	1.000
vga	0 (0.0)	1 (1.2)	0.476
*vga*A	0 (0.0)	0 (0.0)	1.000
vgb	1 (1.1)	0 (0.0)	1.000
RESISTANCE : AMINOGLYSOSIDES			
*aac*A-*aph*D	0 (0.0)	0 (0.0)	1.000
*aad*D	0 (0.0)	0 (0.0)	1.000
*aph*A3	1 (1.1)	0 (0.0)	1.000
RESISTANCE : MISCELLANEOUS GENES			
sat	1 (1.1)	0 (0.0)	1.000
*dfr*A	0 (0.0)	0 (0.0)	1.000
*far*1	1 (1.1)	0 (0.0)	1.000
Q6GD50 (putat. fusidic acid resist.)	3 (3.4)	0 (0.0)	0.247
*mup*R	0 (0.0)	0 (0.0)	1.000
*tet*K	3 (3.4)	1 (1.2)	0.622

*tet*M	0 (0.0)	1 (1.2)	0.476
cat (total)	1 (1.1)	1 (1.2)	1.000
cfr	0 (0.0)	0 (0.0)	1.000
*fex*A	0 (0.0)	0 (0.0)	1.000
RESISTANCE : EFFLUX SYSTEMS			
*qac*A	3 (3.4)	2 (2.5)	1.000
*qac*C (total)	4 (4.5)	5 (6.2)	0.738
RESISTANCE : GLYCOPEPTIDES			
*van*A	0 (0.0)	0 (0.0)	1.000
*van*B	0 (0.0)	0 (0.0)	1.000
*van*Z	0 (0.0)	0 (0.0)	1.000
VIRULENCE : ENTEROTOXINS			
sek	2 (2.2)	3 (3.7)	0.670
sel	15 (16.9)	12 (14.8)	0.834
sem	50 (56.2)	56 (69.1)	0.113
sen	52 (58.4)	56 (69.1)	0.155
seo	49 (55.1)	56 (69.1)	0.082
seq	2 (2.2)	3 (3.7)	0.670
ser	1 (1.1)	4 (4.9)	0.193
seu	52 (58.4)	54 (66.7)	0.342
ORF CM14	2 (2.2)	0 (0.0)	0.498
VIRULENCE : HLG AND LEUKOCIDINS			
*luk*F (*hlg*B)	89 (100.0)	81 (100.0)	1.000
*luk*S (*hlg*C)	89 (100.0)	81 (100.0)	1.000
*hlg*A	86 (96.6)	81 (100.0)	0.247
*luk*F-P83/*luk*M	0 (0.0)	4 (4.9)	0.050
*luk*D	51 (57.3)	41 (50.6)	0.442
*luk*E	36 (40.4)	40 (49.4)	0.281
VIRULENCE : HLB-CONV PHAGES			
sak	64 (71.9)	68 (84.0)	0.067
scn	78 (87.6)	74 (91.4)	0.465
VIRULENCE : EXFOL.TOXINS			
etd	2 (2.2)	5 (6.2)	0.260
VIRULENCE : EPITHEL. DIFF. INHIB			
*edin*A	0 (0.0)	0 (0.0)	1.000
*edin*B	2 (2.2)	7 (8.6)	0.088
edinC	0 (0.0)	0 (0.0)	1.000
VIRULENCE : ACME LOCUS			
ACME	0 (0.0)	0 (0.0)	1.000
VIRULENCE : PROTEASES			
aur	89 (100.0)	80 (98.8)	0.476
*spl*A	53 (59.6)	41 (50.6)	0.281

*spl*B	53 (59.6)	41 (50.6)	0.281
*spl*E	41 (46.1)	42 (51.9)	0.539
CAPSULE -ASSOCIATED GENES			
capsule type 1	0 (0.0)	0 (0.0)	1.000
capsule type 5	37 (41.6)	41 (50.6)	0.281
capsule type 8	52 (58.4)	40 (49.4)	0.281
ADHESION FACTORS / MSCRAMM GENES			
ebh (cons)	89 (100.0)	78 (96.3)	0.106
eno	89 (100.0)	81 (100.0)	1.000
*sas*G (total)	46 (51.7)	38 (46.9)	0.543
vwb (total)	89 (100.0)	81 (100.0)	1.000

[a]IE and non-IE isolates were collected in 2008 and 2006, respectively.
[b]only the genes not shown in Table 1 are presented excluding SCC*mec*-related genes.
[c]P-values were calculated for each gene or allele using a two-tailed Fisher's exact test.