



Staphylococcus aureus colonisant / Staphylococcus aureus infectant dans le modèle du pied diabétique

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THÈSE

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Docteur

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Spécialité : **Microbiologie**

Présentée par **Nourreddine MESSAD**

Staphylococcus aureus colonisant /
Staphylococcus aureus infectant
dans le modèle du pied diabétique

Soutenue le 11 janvier 2016 devant le jury composé de :

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RESUME

Staphylococcus aureus est l'un des principaux agents étiologiques des infections suppuratives superficielles et profondes ainsi que des syndromes liés à l'action de toxines. Paradoxalement, cette bactérie est un agent commensal présent sur la peau ou au niveau de muqueuses (cavités nasales notamment). Les bases génétiques expliquant la différence entre *S. aureus* infectant et *S. aureus* colonisant restent inconnues. Les travaux réalisés au cours de cette Thèse ont permis de mettre en évidence que les souches colonisantes de *S. aureus* appartenaient aux clones CC8/CC5; que les gènes de la famille *edin* permettaient de prédire la mauvaise évolution d'une plaie colonisée; et que les souches de *S. aureus* responsables d'ostéites appartenaient majoritairement au clone ST398. Dans le but de déterminer l'organisation génomique des souches colonisantes, nous avons utilisé le système des Optical maps. Nous avons ainsi déterminé que ces souches appartenaient à un clone différent des souches infectantes. Nous avons identifié, localisé et caractérisé une insertion génétique d'ADN présentes uniquement chez les souches colonisantes. Le séquençage de la zone a permis d'objectiver l'insertion d'un phage au niveau de la région hotspot Φ NM2 en amont du locus *isd*, le principal déterminant du transport membranaire du Fer. Cette insertion avait un impact sur l'activité de Fur, régulateur de l'acquisition du fer, de la virulence et du biofilm chez *S. aureus*. L'expulsion du phage permettait de restaurer la régulation du locus *isd*, la production de sidérophores, la formation de biofilm et la virulence bactérienne. La mutation du gène *fur* nous a permis de déduire que le phage ROSA affectait les bactéries de manière indépendante de Fur. Enfin, nous avons étudié la prévalence des souches colonisantes sur les plaies de pieds diabétiques. Nous avons observé que 20% des souches présentaient l'insertion ROSA et 89% appartenait au complexe clonal CC8. Les souches colonisantes, avec leur niveau bas de virulence, devraient faire l'objet de détection dans le but de rationaliser l'utilisation des antibiotiques et ainsi lutter contre l'apparition de bactéries multiresistantes aux antibiotiques.

Mots-clés : pied diabétique, *Staphylococcus aureus*, virulence, colonisation, infection, marqueurs génétiques diagnostiques, bacteriophage, métabolisme du fer.

SUMMARY

Staphylococcus aureus is an opportunistic bacterium capable of causing a wide range of severe diseases when it gains access to underlying tissues. Paradoxically, this causative pathogen is a common inhabitant of the skin microflora and colonizes the nares and other human mucosa, and as such, may be considered as a commensal colonizing organism. The genetic basis for the difference in pathogenic/colonizing potential remains unknown. During this PhD, we determined that the colonizing strains belonged to CC8/CC5 clonal complexes; the *edin* genes allow to predict the worsening evolution of the colonizing wound; and the *S. aureus* strains isolated from osteitis belonged mainly to ST398 clone. By performing optical maps comparisons of a collection of *S. aureus* strains of defined virulence potential isolated from DFU at different stages, we brought to light a prophage present in colonizing-causing bacteria. The phage, namely ROSA, was localized in a hotspot region Φ NM2 near the locus *isd*, the main iron surface determinant that transport iron across the bacterial wall. It induces a deregulation of the activity of the transcriptional regulator Fur involving the biofilm formation of the bacteria in response to low iron environment. It reduced also significantly the virulence of the strain. The expulsion of the phage restored the regulation of the locus *isd*, the siderophore production, the biofilm formation and the virulence of the strain. The mutation of the *fur* gene within the colonizing strain enabled us to determine that the phage ROSA affected the bacteria in a Fur-independent manner. Finally we determined the prevalence of these colonizing strains in DFU. We observed that 20% of the strains harboured this insertion and 89% belonged to the clonal complex CC8. This colonizing strain by its low virulence potential must be detected in the aim to contribute to a sounder use of antibiotic treatment, an important point in front of the increase of multidrug resistant bacteria.

Keywords: diabetic foot ulcers, *Staphylococcus aureus*, virulence, colonization, infection, diagnostic and genetic tools, bacteriophage, iron metabolism.

ABBREVIATIONS

ADA : American Diabetes Association

CC : Complexe Clonal

CHU : Centre Hospitalier Universitaire

Eap : Extracellular Adherence Protein

Entred : Echantillon national témoin représentatif des personnes diabétiques

HGPO : Hyerglycémie provoquée par voie orale

HMJ : Hyperglycémie modérée à jeun

ICAM-1 : Intercellular Adhesion Molecule 1

InVS : Institut de veille sanitaire

ITG : Intolérance au glucose

LPV : Leucocidine de Panton-Valentine

OMS : Organisation Mondiale de la Santé

PCR : Polymerase Chain Reaction

PFT : Pore-Forming Toxins

PHRC : Programme Hospitalier de Recherche Clinique

RM : Ratio de mortalité

SARM : *Staphylococcus aureus* résistant à la méticilline

SCC*mec* : Staphylococcal Cassette Chromosome *mec*

SERAM : Secretable Expanded Repertoire Adhesive Molecules

TSST-1 : Toxic Shock Syndrom Toxin 1

UFC : Unités Formant Colonie

EDIN : Epidermal Differentiation INhibitor

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I- LE DIABETE SUCRE

1) Définition du diabète sucré et de ses complications

A) Définition

Le diabète sucré est défini comme un groupe d'affections métaboliques caractérisées par une hyperglycémie chronique résultant d'un défaut de la sécrétion ou de l'action de l'insuline ou de ces deux anomalies associées (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus). L'hyperglycémie chronique est associée à terme à des complications organiques spécifiques de la maladie diabétique.

A plusieurs reprises (1979, 1980, 1985 et 1997), les critères diagnostiques du diabète ont été modifiés par les groupes d'experts. La dernière révision a été présentée par un groupe international d'experts à l'initiative de l'« *American Diabetes Association* » (ADA) en 1997, celle-ci a été reprise en 1999 par l'Organisation Mondiale de la Santé (OMS). A l'heure actuelle, un consensus international permet de définir uniformément les anomalies de la tolérance glucidique

B) Classification

Les différentes classes étiologiques de diabète sucré proposées par l'ADA et l'OMS (Drouin et al, 1999) comprennent le diabète de type 1, le diabète de type 2, le diabète gestationnel et les autres types de diabètes sucrés.

Le **diabète de type 1** représente environ 6% des cas de diabètes traités (Figure 1). Il correspond à la destruction des cellules β des îlots de Langerhans du pancréas aboutissant à une carence absolue en insuline. Il est divisé en deux sous-types :

(i) le diabète de type 1 auto-immun : il survient généralement chez le sujet jeune mais il peut apparaître à tous les âges. La destruction des cellules β par un processus auto-immun est authentifiée par la présence d'anticorps anti-cellules d'îlots, anti-insuline, anti-glutamate décarboxylase (GAD), anti-tyrosine phosphatase IA-2 et IA-2b. Cette destruction des cellules β peut être rapide (enfants et adolescents) ou plus lente (adultes). D'autres affections auto-immunes peuvent être associées.

(ii) le diabète de type 1 idiopathique : c'est une forme à forte composante héréditaire. Il concerne une minorité de personnes ; il est plus fréquent chez les sujets d'origine africaine ou asiatique.

Le **diabète de type 2** représente la majorité des cas de diabètes traités (Figure 1) avec un taux de 90%. Il se présente sous forme d'une insulino-résistance hépatique et périphérique, associée à une insulino-pénie relative et progressive. La persistance d'une insulino-sécrétion endogène a conduit à appeler ce diabète « non insulino-dépendant » car l'insulinothérapie n'est pas indispensable à la survie du patient, du moins au début de la maladie.

Le **diabète gestationnel** correspond aux troubles de la tolérance glucidique, de sévérité variable, débutant ou diagnostiqué pendant la grossesse quel que soit le traitement et l'évolution dans le post-partum.

Les **autres diabètes** sont liés à des défauts génétiques de la fonction de la cellule β , liés à des défauts génétiques de l'action de l'insuline, les diabètes induits par une pancréatopathie, les diabètes iatrogènes, les diabètes induits au cours d'infections, les formes rares de diabètes liées à une pathologie du système immunitaire ou encore les diabètes accompagnant divers syndromes génétiques.

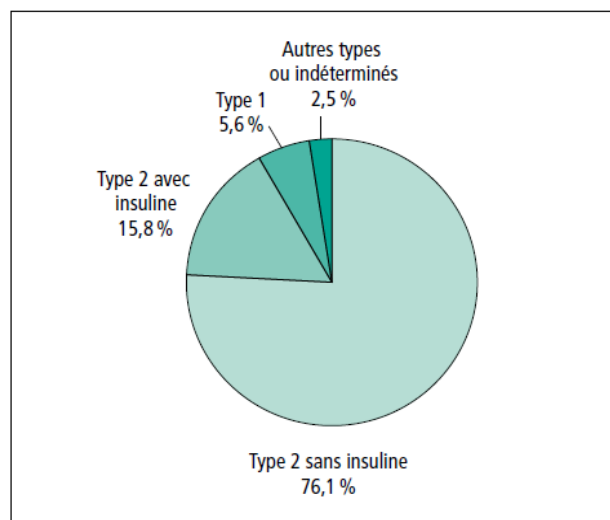


Figure 1 : Distribution des différents types de diabètes selon l'étude Entred 2007-2010 (Fagot-Campagna et al, 2010).

C) Critères diagnostiques du diabète sucré

Une glycorégulation normale se définit par :

- une glycémie à jeun $< 1,10$ g/l
- et**
- une glycémie 2 h après une charge de 75 g de glucose lors d'une hyperglycémie provoquée par voie orale (HGPO) $< 1,40$ g/l

Le diagnostic de diabète peut être établi de trois façons différentes, qui, en l'absence d'une hyperglycémie évidente devront être confirmées par une deuxième mesure :

- symptômes de diabète (polyurie, polydipsie, amaigrissement inexpliqué, somnolence voire coma) et glycémie quelle que soit l'heure $\geq 2,00$ g/L (11,1 mmol/L),
- ou**
- glycémie à jeun $\geq 1,26$ g/L (7,00 mmol/L),
- ou**
- glycémie 2 h après une charge en glucose (HGPO 75 g) $\geq 2,00$ g/L (11,1 mmol/L)

En cas de discordance des deux mesures préconisées pour le diagnostic positif de diabète, une surveillance est généralement conseillée avec contrôles glycémiques périodiques, *a fortiori* en présence de facteurs favorisants (histoire familiale de diabète, âge, obésité, maladies associées).

Ces critères diagnostiques permettent non seulement de définir le diabète sucré mais aussi les anomalies modérées de la tolérance glucidique parmi lesquelles :

- l'intolérance au glucose (ITG) définit par une glycémie 2 heures après une charge en glucose (HGPO 75 g) $\geq 1,40$ et < 2 g/l
- l'hyperglycémie modérée à jeun (HMJ) définit par une glycémie à jeun $\geq 1,10$ et $< 1,26$ g/l.

Les sujets présentant une ITG ou une HMJ sont considérés comme ayant une augmentation du risque de progression vers le diabète. Ces deux catégories de patients ont une glycorégulation qualifiée de « glycorégulation anormale » (Drouin et *al*, 1999 ; HAS Diabètes 2010)

D) Les complications du diabète sucré

L'Institut de veille sanitaire (InVS) avec l'aide de la Caisse nationale d'assurance maladie ont aidé à la réalisation d'études permettant de décrire, d'évaluer et de surveiller l'état de santé des personnes diabétiques traitées. En contre partie, les modalités et les résultats de la prise en charge des diabétiques ont été effectués par l'Institut de veille sanitaire (InVS) avec l'aide de la Caisse nationale d'assurance maladie.

Le contrôle des facteurs de risque vasculaire et de la fréquence des complications chez les personnes diabétiques a été étudiée à partir des études « Entred » (Échantillon national témoin représentatif des personnes diabétiques).

Une première étude « Entred » a montré une qualité de prise en charge médicale insuffisante et un risque vasculaire élevé en 2001 (Fagot-Campagna et al, 2003). Des actions visant à une meilleure prise en charge médicale ont, par la suite, été entreprises au niveau national. C'est pourquoi une deuxième étude « Entred » a été promue, six ans après la première.

« Entred 2007-2010 » a montré une amélioration importante du contrôle des facteurs de risque vasculaires des personnes diabétiques mais également une légère augmentation de la prévalence des complications (Fagot-Campagna et al, 2009). Environ 70% des patients diabétiques de type 2 présentent des complications à plus au moins long terme (Figure 2).

Complications	Prévalence 2007	Intervalle de confiance à 95%	Effectif estimé sur 2,2 millions*
Cardiovasculaires			
Angor ou infarctus du myocarde**	16,7	[15,5 - 18,0]	367 000
Revascularisation coronaire**	13,9	[12,8 - 15,0]	306 000
Insuffisance cardiaque***	6,3	[5,2 - 7,4]	139 000
Accident vasculaire cérébral***	5,0	[4,0 - 5,9]	110 000
Ophtalmologiques			
Perte de la vue d'un œil**	3,9	[3,3 - 4,6]	86 000
Traitement ophtalmologique par laser**	16,6	[15,4 - 17,9]	365 000
Podologiques			
Mal perforant plantaire**	9,9	[8,9 - 10,9]	218 000
Amputation**	1,5	[1,1 - 1,8]	33 000
Rénales			
Dialyse ou greffe**	0,3	[0,1 - 0,4]	9 000

* Sur la base de 2,4 millions de personnes diabétiques traitées en métropole (1), il a été estimé que 91,9%, soit 2,2 millions avaient un diabète de type 2 selon les données d'Entred 2007.
 ** Données pondérées issues de l'auto-questionnaire postal.
 *** Données pondérées issues du questionnaire médecin-soignant.

Figure 2 : Prévalence des complications du diabète de type 2 et nombre de personnes diabétiques atteintes par ces complications en France métropolitaine d'après l'étude Entred 2007 (n = 3894) (Fagot-Campagna et al, 2009).

Ces complications sont la conséquence d'une hyperglycémie chronique. L'hyperglycémie chronique est responsable de perturbations précoces de la circulation avec sur le plan fonctionnel : une

augmentation du débit, de la pression et de la perméabilité capillaire, une perte de l'autorégulation hémodynamique avec vasoplégie artériolaire d'amont, une tendance thrombogène avec notamment une augmentation du facteur de Willebrand synthétisé par les cellules endothéliales et une augmentation de la viscosité sanguine. Par conséquent, le risque d'obstruction de micro-vaisseaux (localisés au niveau de l'œil, du rein...) et de macro-vaisseaux sanguins (localisés au niveau du cœur, du cerveau, des membres inférieurs...) est augmenté.

La **microangiopathie** désigne l'atteinte des petits vaisseaux (artérioles et capillaires). Les complications microangiopathiques comprennent :

- des complications ophtalmologiques : la rétinopathie diabétique correspond à l'atteinte des vaisseaux de la rétine. Dans les pays développés, la rétinopathie diabétique reste la première cause de cécité chez les sujets de 20 à 60 ans.
- des complications rénales : la néphropathie correspond à la détérioration progressive des reins qui se manifeste par divers troubles allant d'une majoration de la micro albuminurie à l'insuffisance rénale.
- des complications neurologiques : la neuropathie diabétique correspond à l'atteinte du système nerveux périphérique et du système nerveux végétatif. La gravité de cette complication est surtout liée aux conséquences cliniques qu'elle entraîne (troubles trophiques, douleurs neuropathiques, atteintes dysautonomiques sévères). La polynévrite diabétique est une des formes cliniques les plus fréquentes et doit systématiquement être recherchée car elle joue un rôle majeur dans l'apparition des lésions des pieds.

La **macroangiopathie** est définie comme l'atteinte des artères de moyen et gros calibre. Elle regroupe les atteintes des artères coronaires, des artères à destinée cervicale et des artères des membres inférieurs. Par conséquent, les complications macroangiopathiques comprennent : l'ischémie coronarienne, l'insuffisance cardiaque, l'artériopathie des membres inférieurs et l'accident vasculaire cérébral.

2) Les chiffres du diabète sucré

La progression mondiale du diabète a été qualifiée d'épidémie par l'OMS (King *et al*, 1998). La réunion des Nations Unies sur les maladies non transmissibles en septembre 2011 a révélé que 356 millions de personnes sont diabétiques dans le monde. Selon les projections de l'OMS, le nombre de décès par diabète va doubler entre 2005 et 2030. Cela ferait du diabète la 7ème principale cause de décès dans le monde.

En France, en 2009, 2,9 millions de personnes étaient traités pour un diabète soit une prévalence du diabète traité de 4,4%. Cette prévalence a augmenté de plus de 6% par an entre 2000 et 2009 (Ricci *et al*, 2010). Le nombre de diabétiques traités en 2009 (2,9 millions) est supérieur à l'hypothèse la plus haute prévue par certains experts pour 2016 (Bonaldi *et al*, 2006). Cela est lié à la progression du surpoids et de l'obésité, à l'intensification du dépistage de la maladie, au vieillissement de la population et à l'amélioration de l'espérance de vie des personnes traitées pour le diabète.

Le Bulletin Epidémiologique Hebdomadaire du 9 novembre 2010 (n°42-43) a présenté une actualisation des données concernant le diabète sucré en France entre 2000 et 2009 à partir des données de l'assurance maladie. Cette étude a permis, entre autre, de mesurer les disparités géographiques et d'estimer l'évolution de la mortalité liée à cette pathologie. L'analyse des taux de prévalence par région en métropole, après standardisation sur l'âge et le sexe, a confirmé les données de 2007 (Kusnik-oinville *et al*, 2007), avec une variabilité géographique importante. Les régions du nord-est de la France se caractérisaient par une forte prévalence du diabète alors que l'ouest, le sud-ouest et le sud-est étaient en grande majorité des régions avec des taux de prévalence beaucoup plus faibles. Les taux de prévalence les plus élevés, en France, étaient observés en outre-mer.

La loi du 9 août 2004 relative à la politique de santé publique concernant spécifiquement le diabète avait pour objectif de réduire la fréquence et la gravité des complications du diabète. Le rapport 2011 sur l'état de santé de la population en France concernant le suivi des objectifs annexés à cette loi de santé publique donnait des précisions sur la prévalence et l'incidence de certaines complications chez les personnes traitées pour diabète (Direction de la recherche, des études, de l'évaluation et des statistiques, rapport 2011)

Sur les 2,4 millions de personnes diabétiques traitées (par antidiabétiques oraux et/ou insuline) vivant en France métropolitaine en 2007, entre 400 000 et 480 000 (environ 20%) auraient subi une complication coronarienne, 94 000 (environ 4%) auraient perdu la vue d'un œil et 7 000 (environ 0,3%) seraient greffées ou dialysées. Entre 19 000 et 36 000 (environ 1%) seraient amputées d'un membre inférieur, entre 55 000 et 240 000 (soit 2 à 10%) auraient un mal perforant plantaire actif ou cicatrisé et entre 110 000 et 415 000 (soit 5 à 17%) auraient bénéficié d'un traitement par laser ophtalmologique.

La présence d'une plaie du pied, *a fortiori* si elle est infectée, est un facteur de surmortalité dans la population diabétique avec un risque de décès multiplié par 2,4 par rapport à un diabétique indemne de plaie (Boyko et *al*, 1996). Entre 1997 et 2007, les taux standardisés d'hospitalisation pour plaie du pied ont presque triplé. Certaines données évaluent que 15 à 25% des patients diabétiques développeront une plaie du pied au moins une fois dans leur vie (Mayfield et *al*, 1998 ; Boulton et *al*, 2005). Secondairement ces plaies s'infectent dans 40 à 80 % des cas (Lipsky, 2001)

Le pronostic des plaies chez le diabétique est dominé par le risque d'amputation. En effet, environ 50% de toutes les amputations des membres inférieurs sont réalisées chez les patients diabétiques qui représentent "seulement" 3 à 5% de la population et dans 70 à 90% des cas, l'amputation est précédée d'une plaie du pied (Dinh & Pham, 2002 ; Moulik & Gill 2002). Les taux d'incidence brut et standardisé de personnes subissant une amputation sont estimés, respectivement, à 376 et 136 pour 100 000 personnes diabétiques, soit 16 fois plus que chez les non-diabétiques. Le niveau d'amputation est le plus souvent celui de l'orteil (43% des cas), devant celui du pied (18%), de la jambe (20%) et de la cuisse (18%). L'étude réalisée dans 38 hôpitaux français en 2010 par Richard et *al*. chez 291 patients diabétiques a confirmé les taux alarmants d'amputations avec 101 patients amputés au cours de l'hospitalisation et 28 dans l'année qui a suivi la prise en charge (soit 48% au total) (Richard et *al*, 2011)

3) Le coût du diabète sucré

De part le statut de maladie chronique, la prise en charge des diabètes de type 1 et 2 représente une source de dépenses non négligeable pour l'Assurance Maladie. Ce coût est sous-estimé du fait des difficultés à évaluer la consommation de soins liée aux besoins indirects engendrés par cette maladie.

L'étude « Entred » réalisée entre 2001-2003 et renouvelée entre 2007-2010 permet de disposer de données sur les aspects économiques du diabète (Fagot-Campagna et *al*, 2009). Ces études ont permis d'analyser les remboursements de l'Assurance Maladie aux personnes traitées pour un diabète sucré en France, de mesurer leur évolution entre 2001 et 2007 et d'identifier les facteurs associés aux consommations de soins. Le remboursement moyen était estimé à 5 300 euros par personne diabétique traitée en 2007. A elles seules, 10% des personnes concentraient 50% des remboursements. Extrapolés à la France entière, les remboursements versés à l'ensemble des personnes diabétiques s'élevaient à 12,5 milliards d'euros, dont 4,7 consacrés à l'hospitalisation faisant de l'hôpital le principal poste de dépense. En analyse multivariée, les facteurs associés aux remboursements de soins étaient :

- l'âge (les patients plus âgés généraient des dépenses deux fois plus élevées que les patients jeunes),
- l'ancienneté de la maladie,
- l'existence de complications micro- et macroangiopathiques,
- le type de traitement médicamenteux (un traitement par insuline revenant plus cher qu'un traitement par antidiabétiques oraux),
- l'existence de comorbidités majeures.

L'augmentation des remboursements (80% entre 2001 et 2007 en euros constants, soit 7,1 à 12,5 milliards) s'explique principalement par l'augmentation du nombre de malades, l'intensification des traitements, l'amélioration du suivi et le changement des pratiques cliniques et thérapeutiques (Robert *et al*, 2009).

II- LE PIED DIABETIQUE

A) Définition du pied diabétique

Le « Pied diabétique » est défini comme l'ensemble des affections atteignant le pied, directement liées aux répercussions du diabète. Celles-ci sont de trois ordres : neurologique, artérielle et infectieuse.

La conjonction de complications de type neurologiques, artérielles et infectieuses favorise l'apparition d'ulcères au niveau du pied chez les personnes diabétiques. En effet, l'absence de douleur, la mauvaise oxygénation des extrémités ainsi que les troubles d'hydratation et la déformation du pied favorisent l'apparition de lésion au niveau du pied. Ces lésions peuvent conduire à l'apparition d'infection. Les infections superficielles concernent les couches tissulaires situées au-dessus de l'aponévrose superficielle et aboutissent à des tableaux de dermohypodermite bactérienne aiguë. Les infections profondes touchent l'aponévrose superficielle (fasciite nécrosante), les tissus mous (abcès) ou les structures ostéoarticulaires (ostéites).

Les bactéries de la microflore commensale cutanée colonisent toutes les plaies chroniques ce qui nécessite de faire la distinction entre une plaie colonisée et une plaie infectée lors de la prise en charge du patient. La colonisation correspond à la présence de microorganismes à la surface de la plaie, sans invasion des tissus et sans réponse immunitaire locale ou générale (absence de signes cliniques ou biologiques d'infection). En revanche, l'infection d'une plaie correspond à la présence de microorganismes réplikatifs dans la plaie avec des signes cliniques et biologiques d'infection résultant de la réponse immunitaire de l'hôte.

B) Mécanismes physiopathologiques de l'infection du pied diabétique

Sur le plan physiopathologique, le premier mécanisme contribuant à favoriser l'infection du pied diabétique est la neuropathie accompagnant le pied diabétique. La neuropathie est une complication précoce et fréquente du diabète, avec des atteintes sensitive, motrice et végétative. L'atteinte sensitive est marquée par la perte de sensibilité thermo-algique responsable de lésions traumatiques indolores. L'atteinte motrice est responsable d'une amyotrophie distale touchant la musculature intrinsèque du pied engendrant des déformations. L'atteinte végétative est responsable de la sécheresse cutanée. Ainsi l'apparition de nouveaux points d'appui associés à une hyperkératose conduit à la formation de zones de frottements qui sont à l'origine de la plaie (Figure 3) (Falanga, 2005).



3a – Pression excessive entraînant la formation d’une hyperkératose réactionnelle



3b – Développement d’une vésicule inflammatoire dans le tissu sous-cutané en regard



3c- Fistulisation à la peau de la vésicule inflammatoire



3d- Mise en communication de l’extérieur avec les tissus et les structures profondes du pied favorisant l’infection de la plaie et le risque d’ostéite.

Figure 3 : Illustration de la formation d’une plaie du pied chez le diabétique (International Working Group on the Diabetic Foot. International consensus on the diabetic foot. 2003. In: International Diabetes Foundation; Brussels. <http://www.iwgdf.org>).

L'artériopathie est le second mécanisme contribuant à la formation d'une plaie du pied et des membres inférieurs liée à une athérosclérose précoce et responsable d'une hypoxie tissulaire. La mort cellulaire et la nécrose tissulaire liées à l'hypoxie créent des conditions optimales à la prolifération microbienne (Bowler *et al*, 2001).

En résumé, les zones d'hyper-appui du pied associées à une sécheresse cutanée et à une ischémie tissulaire distale favorisent la création d'une ulcération du pied.

D'autre part, les patients diabétiques présenteraient un déficit des mécanismes cellulaires de défense majoré par l'hyperglycémie capable d'altérer les fonctions des leucocytes entraînant une moindre efficacité de leur action bactéricide (Richard *et al*, 2012). En effet, l'hyperglycémie aurait un effet néfaste sur le chimiotactisme et la capacité de phagocytose des macrophages et des polynucléaires neutrophiles (Delamaire *et al*, 1997 ; Geerlings & Hoepelman 1999).

Enfin, la chronicité des plaies et l'anatomie particulière du pied en elle-même sont des facteurs favorisant de l'infection (Rauwerda, 2000).

C) Diagnostic d'infection d'une plaie du pied diabétique

1. Classifications cliniques

Le diagnostic d'une infection d'une plaie du pied chez le diabétique est un diagnostic clinique puisque la plaie est obligatoirement colonisée par la flore commensale du patient et la flore environnementale, la présence de bactéries sur une plaie n'est en aucun cas synonyme d'infection.

Le diagnostic repose sur l'observation clinique et en particulier sur la présence d'au moins deux des signes suivants (Lipsky *et al*, 2004):

- augmentation du volume du pied,
- induration,
- érythème périlésionnel,
- sensibilité locale ou douleur,
- chaleur locale,
- présence de pus.

Afin d'aider le clinicien à juger de la sévérité de l'infection, plusieurs classifications cliniques ont été proposées (Jeffcoate *et al*, 1993 ; Van Acker *et al*, 2004). Parmi elles, deux ont été retenues.

La première est la classification de l'université du Texas (Lavery *et al*, 1996 ; Oyibo *et al*, 2001). Elle a été validée sur une étude prospective de cohorte (Armstrong *et al*, 1998). Elle comporte quatre grades en

fonction de la profondeur et quatre stades selon la présence ou non d'une infection et/ou d'une ischémie (Tableau 1). Cependant, dans cette classification, l'infection n'est caractérisée que par sa présence/absence et par la profondeur de l'atteinte.

Une classification complémentaire de l'infection de la plaie a donc été définie dans le Consensus International sur le pied diabétique (Tableau 2). L'infection y est définie par la présence d'au moins deux signes d'inflammation (*cf.* ci-dessus) et sa gravité se décline en 3 grades allant d'une infection légère (grade 2) à une infection sévère (grade 4), en fonction de l'extension de l'infection aux tissus mous et aux annexes et à la présence de signes systémiques et/ou de troubles métaboliques. Cette classification a été validée au cours d'une étude prospective (Lavery et *al*, 2007). Ces critères ont été retenus par la Société de Pathologie Infectieuse de Langue Française en 2007.

Cependant, les manifestations cliniques retenues dans cette classification peuvent être réduites chez le patient diabétique (en raison de l'artériopathie et de la neuropathie sous-jacente). D'autres paramètres sont donc nécessaires pour aider au diagnostic d'infection quand les signes classiques sont absents.

	Grade 0 lésion épithéliale (%)	Grade 1 plaie superficielle (%)	Grade 2 Atteinte du tendon ou de la capsule (%)	Grade 3 Atteinte de l'os ou de l'articulation (%)
Stade A Pas d'infection Pas d'ischémie	0A (0)	1A (0)	2A (0)	3A (0)
Stade B Infection Pas d'ischémie	0B (12,5)	1B (8,5)	2B (28,6)	3B (92)
Stade C Pas d'infection Ischémie	0C (25)	1C (20)	2C (25)	3C (100)
Stade D Infection et ischémie	0D (50)	1D (50)	2D (100)	3D (100)

Tableau 1 : Classification des plaies du pied chez le patient diabétique : Classification UT (University of Texas).

Sévérité de l'infection (IDSA)	PEDIS grade	Critères cliniques
Non infecté	Grade 1	Pas de symptôme, ni de signe d'infection
Légère	Grade 2	Atteinte cutanée uniquement (sans atteinte des tissus sous-cutanés, ni systémique) avec au moins deux des signes suivants : - chaleur locale - érythème > 0,5-2 cm autour de l'ulcère - sensibilité locale ou douleur - tuméfaction locale ou induration - Décharge purulente (sécrétion épaisse, opaque à blanchâtre ou sanguinolente) Les autres causes de réaction inflammatoire de la peau doivent être éliminées (par exemple : traumatisme, goutte, pied de Charcot aigu, fracture, thrombose, stase veineuse)
Modérée	Grade 3	• Erythème > 2 cm et une des constatations décrites ci-dessus ou • Infection atteignant les structures au-delà de la peau et du tissu sous-cutané, comme un abcès profond, une lymphangite, une ostéite, une arthrite septique ou une fasciite. Il ne doit pas y avoir de réponse inflammatoire systémique (cf grade 4)
Sévère ^a	Grade 4	Quelque soit l'infection locale, si présence de signes systémiques (SIRS) manifestées par <u>au moins deux des caractéristiques suivantes</u> : - température > 38° ou < 36°C - fréquence cardiaque > 90 battements/mn - fréquence respiratoire > 20 cycles/mn - PaCO₂ < 32 mm Hg - leucocytes > 12 000 ou < 4 000 /mm³ - ≥10% de formes leucocytaires immatures

IDSA, Infectious Diseases Society of America ; SIRS, syndrome de réponse inflammatoire systémique

^aL'ischémie peut augmenter la gravité de toute infection, et la présence d'ischémie critique rend souvent l'infection sévère. L'infection systémique peut parfois s'associer à d'autres situations cliniques, comme l'hypotension, la confusion, les vomissements, ou des troubles métaboliques, tels que l'acidose, l'hyperglycémie sévère et une azotémie d'apparition récente.

Tableau 2 : Classification de l'infection des plaies du pied selon le Consensus International sur le pied diabétique (International Working Group on the Diabetic Foot) : PEDIS (perfusion, étendue/taille, profondeur (depth)/perte tissulaire, infection, sensibilité).

2) Examens d'intérêt dans la documentation de l'infection du pied diabétique

• Examens microbiologiques

Les prélèvements microbiologiques n'ont d'intérêt que dans le cas d'une infection documentée cliniquement à partir d'un grade 2 de la classification du Consensus International sur le pied diabétique.

Le but des examens microbiologiques standards est d'obtenir l'isolement et l'identification de la ou des bactérie(s) responsable(s) de l'infection à partir d'un prélèvement en évitant sa contamination par la microflore commensale qui colonise la peau.

La Figure 4 résume le choix des prélèvements à effectuer en fonction du type de la plaie. Les prélèvements doivent être réalisés sur une plaie débridée soit de manière chirurgicale (au bloc opératoire par un chirurgien), soit de manière mécanique au moyen d'une curette ou d'un scalpel stériles et nettoyée avec du sérum physiologique.

Concernant l'interprétation des résultats, l'écologie bactérienne des plaies infectées du pied diabétique sera développée dans le paragraphe II-4. Brièvement, il est conseillé dans un premier temps de ne pas tenir compte des bactéries reconnues comme étant les moins virulentes ou comme commensales (staphylocoques à coagulase négative, entérocoques, corynébactéries, *Pseudomonas aeruginosa*) (Lipsky et al, 2004 ; Hartemann-Heurtier et al, 2000). En cas de doute, les prélèvements doivent être répétés et ces bactéries seront prises en considération si elles sont isolées à plusieurs reprises.

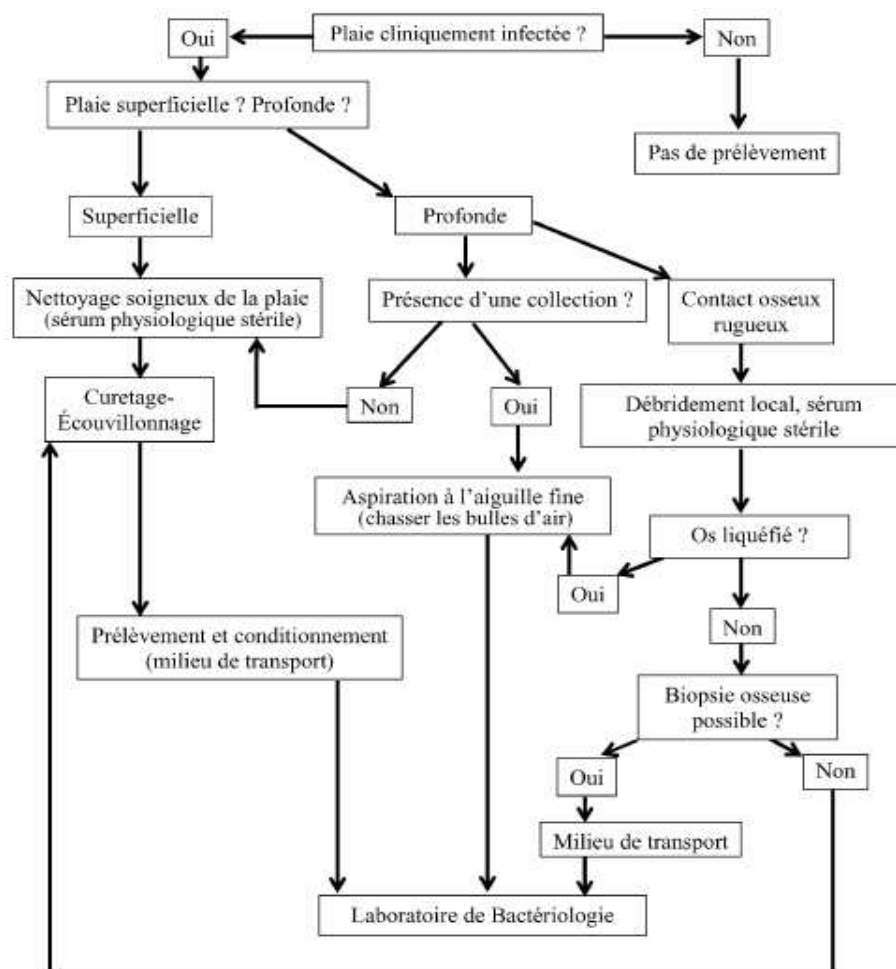


Figure 4 : Prélèvements microbiologiques à effectuer en fonction du type de plaie du pied.

- **Autres examens biologiques**

L'hyperleucocytose ($> 12\,000/\text{mm}^3$) ou la leucopénie ($< 4\,000/\text{mm}^3$) à polynucléaires neutrophiles entrent dans la définition d'une infection sévère mais manquent de spécificité.

Les marqueurs de l'inflammation sont également à prendre en considération. En particulier, la combinaison des taux de CRP et de procalcitonine (PCT) serait la plus discriminante pour différencier les infections des colonisations (Jeandrot et *al*, 2008).

Cependant, les valeurs de ces marqueurs ne sont pas constamment corrélées avec la sévérité de l'infection et manquent de spécificité. De plus, le coût de la PCT est souvent très élevé. Le suivi du taux de la CRP (et sa diminution) est un bon marqueur de guérison de l'épisode infectieux.

- **Examens complémentaires**

En dehors de la composante microbiologique et du déséquilibre glycémique, d'autres facteurs d'aggravation de l'infection sont à rechercher.

Le clinicien va devoir repérer les points d'hyper-appui et l'ensemble des facteurs mécaniques pouvant favoriser la formation d'une plaie (port de chaussures blessantes, pathologie unguéale, déformations acquises, alitement prolongé, œdème,...) afin de les éradiquer. L'évaluation de l'état vasculaire du patient est également indispensable grâce :

- à la clinique (inspection du pied, palpation des poulx, auscultation des trajets vasculaires),
- à des examens complémentaires (à réalisés selon les cas) comme la mesure de l'index de pression systolique (IPS), l'écho-doppler artériel des membres inférieurs, l'artériographie, la mesure de la pression systolique au gros orteil (PSGO) ou encore la mesure transcutanée de la pression en oxygène (TcPO₂).

Enfin, l'imagerie est un outil essentiel afin de diagnostiquer la présence ou non d'une ostéite. Les radiographies simples sont à réaliser en première intention. Les signes évocateurs (réaction périostée, ostéopénie, ostéolyse) peuvent être absents à un stade précoce de l'infection donnant des clichés normaux en début d'infection. L'imagerie devra alors être répétée à distance (2 à 4 semaines). En présence d'une forte suspicion d'ostéite et en l'absence de signes évocateurs radiologiques, d'autres explorations peuvent être envisagées tel que l'IRM ou la scintigraphie.

D) Ecologie bactérienne des plaies infectées du pied diabétique

L'écologie bactérienne des plaies infectées du pied diabétique est le plus souvent polymicrobienne (Ge et al, 2002 ; Citron et al 2007). L'étude OPIDIA, étude prospective menée dans 38 centres hospitaliers français, a permis d'analyser la prise en charge des plaies du pied diabétique infecté en France chez 291 patients. Des prélèvements microbiologiques ont été réalisés chez 86% de ces patients. Les bactéries les plus fréquemment isolées sont les cocci à Gram positif (60%). Parmi ces bactéries, *S. aureus* est la bactérie la plus fréquente (54% des bactéries à Gram positif et 32,5% de l'ensemble des souches isolées). Les streptocoques β -hémolytiques occupent la deuxième position parmi les cocci à Gram positif (14% des souches isolées). Les entérobactéries, les entérocoques et *Pseudomonas aeruginosa* représentent respectivement 27%, 8% et 7% des souches détectées. Les bactéries anaérobies n'étaient isolées que dans 2% des cas. Vingt pourcent des patients étaient concernés par une infection à bactérie multirésistante : 26% par des souches de *Pseudomonas aeruginosa* et 25% par des *S. aureus* (Richard et al, 2011).

En résumé, les bactéries aérobies à Gram positif sont les plus fréquentes. *S. aureus* est, en particulier, la bactérie la plus souvent mise en évidence de façon isolée ou au sein d'une population polymicrobienne dans les pays développés (Ge et al 2002 ; Lipsky et al 2006). Selon les études, *S. aureus* représente 29 à 76% des bactéries isolées dans les infections des plaies du pied diabétique avec 11,5 à 30% de SARM (EL-Tahawy, 2000 ; Goldstein et al, 1996 ; Richard et al, 2008). Les streptocoques β -hémolytiques sont également fréquents (Lavery et al, 1994).

Les bactéries aérobies à Gram négatif isolées appartiennent le plus souvent à la famille des entérobactéries (*Proteus mirabilis*, *Escherichia coli*, *Klebsiella* spp). Elles se rencontrent, généralement, sur des infections chroniques. *P. aeruginosa* est plus souvent isolé dans des cas d'ostéites que dans les infections des tissus mous (Lipsky, 1997). Cette bactérie est volontiers isolée en cas d'hospitalisations prolongées. Son rôle pathogène est cependant discuté. Toutefois, c'est la bactérie la plus fréquemment isolée dans les pays en voie de développement (Asie du Sud Est notamment). Les bactéries anaérobies strictes sont souvent associées à des bactéries aérobies-anaérobies facultatives mais très rarement isolées. *Fingoldia magna* et *Bacteroides* spp. sont les plus pathogènes. Les études par métagénomique des plaies montrent que ces bactéries sont constamment présentes dans des plaies profondes (Gardner SE et al, 2013).

III- *Staphylococcus aureus* ET INFECTION DU PIED DIABETIQUE

1) Généralités

Le genre *Staphylococcus* appartient à la famille des *Micrococcaceae*. Ce genre se compose de cocci à Gram positif groupés en amas, asporulés et aéro-anaérobies facultatifs (Figure 5). Il comprend 44 espèces mais la plus fréquente en pathologie humaine est *S. aureus*. Cette bactérie à comportement commensal se situe dans diverses niches écologiques comme la peau ou la plupart des muqueuses chez les mammifères à sang chaud. Elle est extracellulaire mais peut subsister, dans certaines conditions, dans le milieu intracellulaire (Sendi et Proctor, 2009). Chez l'homme, environ 30% de la population est porteuse chronique de *S. aureus* principalement au niveau des fosses nasales (Rich, 2005 ; Wertheim et al, 2005). A partir de ce site de portage, *S. aureus* colonise les territoires cutanés, en particulier les zones humides (aisselles, plis, périnée) et les mains. La transmission intra ou interhumaine se produit généralement par contact direct (manuportage) et les infections surviennent lors de la rupture de la barrière cutanéomuqueuse. Plus rarement, elle peut être indirecte à partir d'une source environnementale (vêtements, draps, matériels médicaux). Cependant un affaiblissement du système immunitaire peut également conduire à des infections très diverses en brisant le fragile équilibre qui existe entre la bactérie et son hôte. En effet, une fois dans le sang, la bactérie peut facilement coloniser de nombreux organes vitaux et induire des infections sévères parfois mortelles telles que les endocardites, les chocs septiques, les pneumonies ... (Lina et al, 1997). De plus, *S. aureus* est une bactérie pouvant être difficile à soigner car l'utilisation massive d'antibiotiques a induit la sélection, l'émergence et la diffusion de souches multirésistantes aux antibiotiques (Hiramatsu, 2001 ; Hiramatsu et al, 2001). Ainsi, les souches de *S. aureus* résistantes à la méticilline (SARM), même si elles sont actuellement en régression du fait de campagnes nationales efficaces pour l'utilisation des solutions hydroalcooliques, restent un véritable problème dans nos hôpitaux. Comme *S. aureus* est capable de former un biofilm compact qui le protège contre la plupart des agressions extérieures comme l'action des détergents ou des processus de stérilisation, il demeure une des principales bactéries responsables d'un grand nombre d'infections associées aux soins chaque année. La gravité et la fréquence de ces infections en font un problème de Santé Publique.

Le pouvoir pathogène de *S. aureus* est étroitement lié à l'expression de nombreux facteurs de virulence au niveau du site d'infection (Foster, 2004 ; Holden et al, 2004 ; Novick, 2003). Ces facteurs codent pour des protéines de surfaces ou des exoprotéines, et permettent à la bactérie de combattre le système immunitaire, d'adhérer aux cellules, de disséminer dans l'hôte en utilisant l'énergie et les nutriments disponibles (Novick, 2003). Chaque type d'infection nécessite une combinaison particulière de facteurs de virulence. Ainsi, les protéines de surface sont synthétisées en début de phase de croissance puis réprimés en phase exponentielle de croissance alors que la plupart des exoprotéines sont synthétisées en phase post exponentielle (Dunman et al, 2001 ; Vandenesch et al, 1991). Ce mode de régulation alterné mime les étapes d'infection *in vivo* et suggère le modèle suivant : en début d'infection, les bactéries synthétisent prioritairement des adhésines pour adhérer et coloniser l'hôte, puis lorsque les bactéries s'accumulent, la

synthèse des adhésines est inhibée au profit des exoprotéines. Cette réorganisation de l'expression des facteurs de virulence permet à la bactérie de récupérer des nutriments mais surtout de se disperser dans l'hôte en échappant au système immunitaire et de coloniser de nouveaux sites.

Chez *S. aureus*, de nombreux facteurs de virulence ainsi que plusieurs gènes de résistance aux antibiotiques sont codés au niveau d'îlots de pathogénicité (Hochhut et al ; Novick 2003b). Ces régions mobiles rendent le génome très dynamique en favorisant les transferts horizontaux entre différentes souches bactériennes. Ainsi, ces dernières années, le séquençage de différents génomes de *S. aureus* a montré que des souches considérées comme peu pathogènes avaient acquis des gènes de virulence par transfert d'îlot de pathogénicité. Ces îlots expliquent en partie pourquoi *S. aureus* s'adapte à des conditions environnementales multiples.

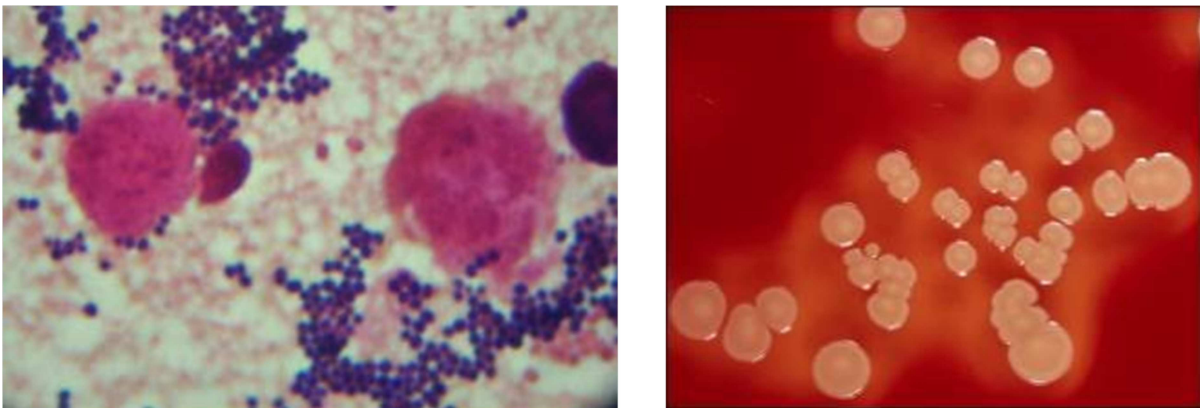


Figure 5 : Aspect caractéristique de *S. aureus* à la coloration de Gram (à gauche) et colonies de *S. aureus* en culture sur gélose au sang (à droite) (<http://www.microbe-edu.org/etudiant/staph.html> http://fundacionio.org/img/bacteriology/cont/Staphylococcus_aureus.html)

2) Facteurs de virulence du *S. aureus* dans les infections cutanées

S. aureus est une bactérie pyogène responsable d'infections suppurées de la peau et des muqueuses; elle est notamment le principal pathogène des plaies chroniques. Ces infections cutané-muqueuses sont parfois le point de départ de suppurations profondes diffusant par voie hématogène et donnant lieu à de fréquentes localisations métastatiques génératrices d'infections profondes (pleuro-pulmonaires, ostéo-articulaires, cardiaques cérébro-méningées, génito-urinaires...). *S. aureus* est aussi responsable d'infections toxiques telles que le syndrome d'exfoliation généralisé, le syndrome de choc toxique staphylococcique, la pneumonie nécrosante ou les toxi-infections alimentaires.

A) Facteurs de virulence associés à la capsule et à la paroi

La paroi et la membrane externe du *S. aureus* participent au pouvoir pathogène de la bactérie. En effet, les acides lipoteichoïques membranaires ainsi que le peptidoglycane aident à l'adhésion du *S. aureus* à la matrice extracellulaire de l'hôte. Des polysaccharides capsulaires sont rencontrés chez 90% des souches. La capsule permet une meilleure résistance des souches à l'opsonisation et à la phagocytose. Parmi les onze types de polysaccharides capsulaires identifiés, les types 5 et 8 (Cap5 et Cap8) sont détectés dans près de 75% des infections humaines.

Enfin, il existe de nombreuses protéines de surface, appelées « adhésines », qui jouent un rôle primordial dans la capacité du *S. aureus* à coloniser les tissus. Ces adhésines comprennent :

- des protéines ancrées à la surface de la bactérie par des liaisons covalentes : c'est la famille des MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). Parmi celles-ci, nous comptons :
 - les protéines de liaison au collagène (Cna) : constituants majeurs de la matrice extracellulaire, elles représentent un site de choix pour l'ancrage de *S. aureus* aux tissus. Elles jouent un rôle important dans les infections ostéo-articulaires.
 - les protéines de liaison au fibrinogène (clumping factor) : sont des récepteurs pour le fibrinogène qui provoquent l'agrégation des bactéries en présence de plasma. Il existe 2 types de protéines : ClfA et ClfB codées par deux gènes distincts et reconnaissant deux régions différentes du fibrinogène.
 - les protéines de liaison à la fibronectine (Fnbp A et B) : permettent à *S. aureus* de se lier à la fibronectine avec une très grande affinité et de façon quasi irréversible. Elles favorisent la colonisation tissulaire et l'adhérence de *S. aureus* aux caillots plasmatiques et aux biomatériaux ayant un contact prolongé avec le sang.
 - la protéine de liaison au facteur von Willebrand ou protéine A (Spa) : la protéine A possède deux domaines de liaison aux immunoglobulines : un fixant le fragment Fc (partie constante) et l'autre le fragment Fab (partie variable). L'interaction protéine A-fragment Fc des immunoglobulines interfère avec l'opsonisation anticorps-dépendant et contribue ainsi au phénomène d'échappement aux défenses de l'hôte (Cheung et al, 2002). L'interaction protéine A-fragment Fab entraîne une activation polyclonale des lymphocytes B. La protéine A se comporte ainsi comme un superantigène pour les lymphocytes B.
 - Les protéines de liaison à l'élastine de *S. aureus* (EbpS) sont des protéines de 25 kDa associées à la surface cellulaire. Elles favorisent la colonisation tissulaire.
- des protéines qui peuvent être associées à la surface de la bactérie ou excrétées : ce sont les protéines de la famille des SERAM (Secretable Expanded Repertoire Adhesive Molecules). En particulier, « Eap » (Extracellular Adherence Protein) est capable de se lier à ICAM-1 (Intercellular Adhesion

Molécule 1), à la surface des cellules endothéliales, inhibant ainsi l'adhésion des leucocytes et leur extravasation du flux sanguin vers le site de l'infection (Haggar et *al*, 2004 ; Chavakis et *al*, 2005).

B) Facteurs de virulence excrétés

1-Les toxines

S. aureus produit de nombreuses toxines qui peuvent être classées par mécanisme d'action :

- les toxines formant des pores ou « pore-forming toxins » (PFT) :

Ces toxines cytolytiques détruisent les cellules de la défense de l'hôte en formant des pores au niveau des membranes cellulaires (Parker & Feil, 2005). On distingue deux grands groupes de PFTs en fonction de leur structure tridimensionnelle (Prévost et *al*, 2001)

Le premier groupe est formé par les alpha-PFTs, dont la structure secondaire est formée d'une simple hélice alpha hydrophobe. Ce groupe comprend la delta-hémolysine qui possède des propriétés détergentes.

Le second groupe est formé par les bêta-PFTs, où le pore est constitué majoritairement de brins bêta s'assemblant en tonneaux bêta. Les cibles cellulaires majeures des bêta-PFTs sont les leucocytes (polynucléaires neutrophiles, monocytes, macrophages et lymphocytes), d'où l'emploi du terme de leucotoxines ou de leucocidines pour en désigner la majorité (Parker & Feil, 2005). Ce groupe comprend :

i) l'alpha-hémolysine, toxine ayant pour cible les cellules endothéliales, les monocytes et les thrombocytes. La destruction des cellules endothéliales favorise la dissémination des bactéries et les métastases septiques à distance.

ii) des leucotoxines à deux composés avec, notamment, la leucocidine LukD-LukE, la leucocidine de Panton-Valentine (LPV) et la gamma-hémolysine (Hlg).

Le gène codant pour LukD-LukE est présent dans près de 90% des souches, mais il n'est exprimé que dans seulement un tiers des souches. LukD-LukE a été isolée chez plus de 75% des souches de *S. aureus* responsables d'impétigo bulleux, produisant également les épidermolysines A ou B (Gravet et *al* 2001). Son rôle dans la pathogénicité reste toutefois controversé.

Le locus codant pour la production de la LPV est porté par un phage et n'est détecté que dans un nombre restreint de souches de *S. aureus* en France (< 2%) (Kaneko et *al*, 1998). Cette LPV a une action leucotoxique et dermonécrotique. Il a été montré que 90% des souches produisant la LPV sont issues de furoncles, et inversement que 96% des cas de furoncles sont associés à des souches productrices de LPV (Couppie et *al*, 1994). La LPV est également associée à des infections profondes sévères : essentiellement des pneumopathies communautaires nécrosantes et hémorragiques chez de jeunes adultes (Labandeira-Rey et *al*, 2007) mais aussi des ostéomyélites (Gillet et *al*, 2007).

La gamma-hémolysine se fixe sur les érythrocytes mais aussi sur les leucocytes. Elle est donc hémolytique et leucotoxique. Une forte proportion des souches de *S. aureus* produit la gamma-toxine (99%).

- les toxines à activité anti-protéolytique

S. aureus produit des inhibiteurs de protéases qui ont une action « anti-complément » :

- la « chemotaxis inhibitory protein of *Staphylococcus aureus* » (CHIPS) : action anti-C5a avec pour conséquence une action inhibitrice du chimiotactisme des neutrophiles et des monocytes,

- les « staphylococcal complement inhibitor » (SCIN) : inhibiteurs des C3 convertases.

Ces toxines ont pour conséquence de minimiser l'opsonisation des bactéries et de limiter la mise en place d'une défense immunitaire adaptée.

2-Les enzymes

S. aureus produit de nombreuses enzymes comme des protéases, lipases, hyaluronidases qui lysent les tissus et peuvent faciliter l'extension de l'infection aux tissus adjacents.

La coagulase se lie à la prothrombine et forme un complexe appelé staphylothrombine. Ce complexe entraîne la polymérisation du fibrinogène en fibrine et la formation d'un caillot protégeant la bactérie de la phagocytose (Kawabata & Iwanaga, 1994).

La staphylokinase (sak), est un activateur du plasminogène en plasmine et exerce un effet inverse à celui de la coagulase. Il conduit à la dislocation du thrombus qui est très riche en bactéries et ainsi à la formation de localisations septiques secondaires.

S. aureus produit également une désoxyribonucléase thermostable : nucléase ayant des propriétés endo- et exonucléasiques.

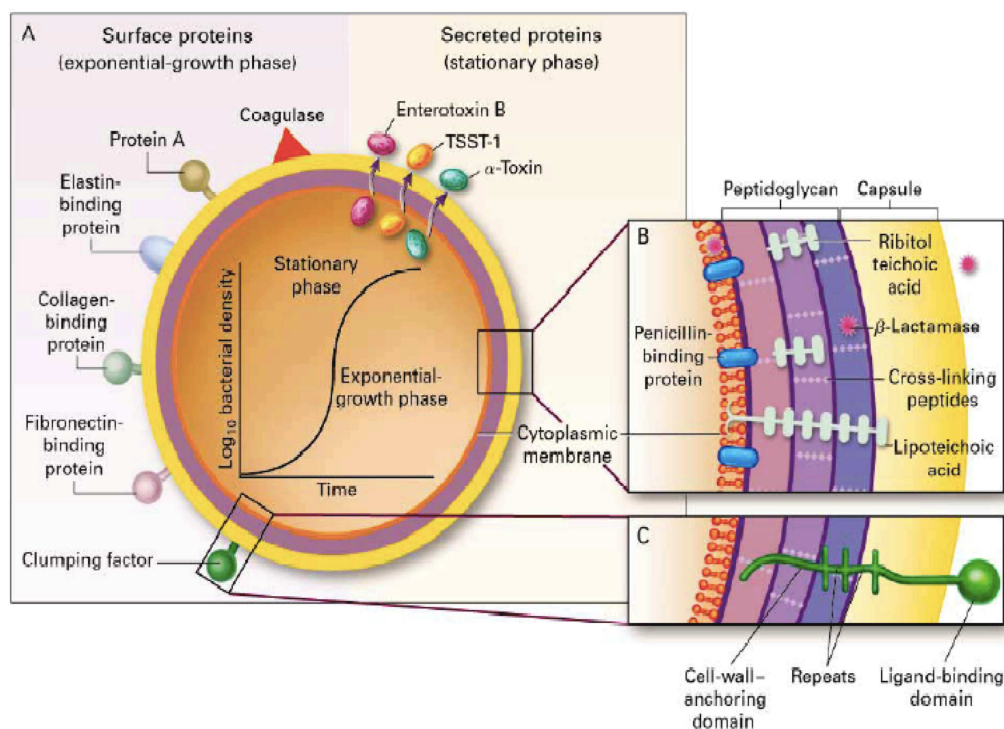


Figure 6: Structure de *S. aureus* (Jones, 1998)

A : Protéines de surface et leurs sécrétions en fonction de la phase de croissance. B : Organisation de la paroi bactérienne et de la capsule. C : Détail de l'organisation du « clumping factor ». La majorité des protéines de surfaces ont une organisation similaire.

C) Organisation en biofilm

Le biofilm est une communauté mono ou polymicrobienne fixée à une surface naturelle ou synthétique, solide ou non, et maintenue par la sécrétion d'une matrice adhésive et protectrice lui conférant une structure tridimensionnelle. C'est une structure vivante, dynamique, en perpétuel remaniement (Sutherland, 2001). Les bactéries planctoniques quittent périodiquement le biofilm, se multiplient et se dispersent dans la circulation conduisant à un processus d'inflammation chronique et récidivant. Le développement d'un biofilm est sous l'influence de signaux extracellulaires (environnementaux) et cellulaires (notion de quorum sensing).

Les biofilms sont responsables d'un large éventail d'infection chez l'homme. Grâce à la technique de microscopie électronique à balayage, James *et al.* ont montré que 60% des plaies chroniques, parmi lesquelles se trouvaient des plaies infectées du pied chez des patients diabétiques, étaient concernées par la présence de biofilm (James *et al.*, 2008).

Or, les staphylocoques sont responsables de la grande majorité des infections causées par des biofilms (Otto, 2008). Une étroite corrélation entre virulence et capacité à former des biofilms a été démontrée chez

S. aureus (Otto, 2008 & Götz, 2002). De multiples facteurs pathogéniques chez cette bactérie contribuent aux différentes phases de formation du biofilm (Otto, 2008):

- les protéines de la famille des MSCRAMM sont nécessaires à la phase d'adhésion,
- la « polysaccharide intercellular adhesin » (PIA) est une molécule indispensable dans la phase de maturation du biofilm pour permettre l'adhésion intercellulaire,
- les « Phenol-soluble modulins » (PSM) sont des peptides avec un effet surfactant qui aide à structurer le biofilm. Les PSM ont également la capacité de tuer rapidement les polynucléaires neutrophiles et d'activer l'inflammation.

Le mode de vie sous forme de biofilm apporte plusieurs avantages à cette bactérie (Costerton et *al*, 1999):

- avantages métaboliques : économie d'énergie et constitution de réserves,
- protection mécanique : les micro-organismes sont préservés d'un certain nombre de facteurs d'agression comme par exemple la dessiccation, l'action d'agents antibiotiques, la réponse immunitaire de l'hôte,
- acquisition de caractères de résistance : l'organisation en communauté de micro-organismes au sein du biofilm est propice à l'échange d'informations, avec entre autres, la possibilité de transfert de caractères de résistance. Ainsi, la concentration minimale inhibitrice d'un antibiotique vis-à-vis d'une bactérie quiescente (en biofilm) peut être 100 à 1000 fois supérieure à celle du même antibiotique vis-à-vis de la même bactérie sous forme planctonique (Høiby et *al*, 2010).

L'adaptation des biofilms à des conditions environnementales stressantes explique l'acquisition d'avantages évolutifs par les micro-organismes qui le constituent.

D) Autres Facteurs de virulence

L'EDIN (Epidermal Differentiation INhibitor) est un facteur de virulence qui confère des propriétés invasives à *S. aureus*. Trois types d'EDIN ont été identifiés à ce jour : EDIN-A, EDIN-B, et EDIN-C. Un travail récemment publié a démontré qu'une souche de *S. aureus* isolée d'une bactériémie associée à une spondylodiscite produit la formation de larges tunnels ou "macroapertures" (MAs) au travers des cellules endothéliales *in vitro* et *ex vivo* (Boyer L et *al*, 2006)

). L'ouverture des MAs dans le tapis cellulaire endothélial rend possible la fixation des staphylocoques au niveau de la matrice extracellulaire. Cette capacité est conférée par le facteur EDIN. L'EDIN induit aussi la formation de MAs dans des cellules fibroblastiques et ostéoblastiques (Boyer L et *al*, 2006). Son mode d'action moléculaire est bien décrit. EDIN appartient à la famille des exoenzymes de la famille C3 décrites chez *Clostridium botulinum* (Aktories K et *al*, 2004)

). Ces facteurs inactivent la protéine cellulaire RhoA par ADP-ribosylation. RhoA est un membre de la famille des protéines GTPases Rho, régulateurs centraux de l'organisation et de la dynamique du cytosquelette d'actine. RhoA en activant la Rho kinase induit de façon directe et indirecte la phosphorylation et l'activation de la sous-unité régulatrice de la chaîne légère de la myosine, avec comme conséquence la formation de câbles d'actine et leurs contractions. L'inhibition ou la déplétion de RhoA après ADP-ribosylation par le facteur EDIN conduit à une dissolution de ces câbles d'actine et l'induction de MAs, notamment dans les cellules endothéliales (Boyer L *et al*, 2006). L'analyse de ces structures par vidéomicroscopie a permis d'établir, qu'*in vitro*, l'ouverture de ces tunnels intracellulaires est transitoire. Enfin, l'analyse de l'effet de l'EDIN sur des monocouches de cellules endothéliales et dans un modèle animal indique que l'ouverture des macroapertures s'associe à l'induction par l'EDIN d'une perméabilité vasculaire. L'EDIN est donc un facteur bactérien capable de produire une interruption de la barrière endothéliale. Cette découverte soulève de nombreuses questions, en particulier la place du facteur EDIN dans la virulence des souches de *S. aureus* et dans la gravité des infections ; un tel mécanisme pourrait être impliqué dans le développement des métastases septiques à *S. aureus*, en conférant à la bactérie un mode d'invasion des tissus jusqu'ici inconnu. Très récemment EDIN-B a été impliqué dans la translocation de cette bactérie dans le sang lors des pneumonies à SARM communautaires (Courjon *et al*, 2015).

E) *S. aureus* et le Fer

1-La capacité à assimiler le fer dans l'environnement

Les vertébrés restreignent l'accès aux nutriments essentiels dans ce qu'on appelle maintenant l'immunité nutritionnelle. La séquestration du fer par l'hôte est l'archétype de ce genre d'immunité nutritionnelle. Ainsi, quel que soit l'environnement dans lequel se trouve la bactérie, le fer est essentiel pour sa physiologie, notamment pour les mécanismes de transport d'électrons, la synthèse des nucléotides, certains métabolismes enzymatiques et respiratoires et, donc la croissance bactérienne (Braun, 2001; Wandersman & Delepelaire, 2004).

Dès l'entrée dans un hôte, une bactérie pathogène doit acquérir du fer à partir des tissus pour survivre (Wandersman & Delepelaire, 2004).

Les bactéries peuvent s'approvisionner en fer selon cinq stratégies différentes (Griffiths *et al*, 1988; Guérinot, 1994; Ratledge & Dover, 2000) :

- à partir des produits de dégradation de l'hémoglobine après hémolyse ;
- directement à partir de la transferrine ou de la lactoferrine de l'hôte ;
- indirectement à partir de ces deux mêmes molécules par la production de sidérophores ;

- indirectement à partir de sidérophores produits par d'autres bactéries ;
- à partir des pools intracellulaires de fer pour les bactéries pathogènes intracellulaires facultatives et obligées.

S. aureus a développé plusieurs systèmes sophistiqués d'absorption du fer. Comme pour nombre de bactéries, sa capacité à acquérir le fer au cours de l'infection est très importante pour sa virulence (Torres et al, 2006).

2-Réservoir du fer chez l'hôte

Le fer est présent en grande quantité dans le sang et les tissus de l'homme, mais le fer libre, sous forme d'ions ferreux, seul utilisable par les bactéries, n'est pratiquement pas disponible chez l'hôte ou en très faibles quantités (10^{-18} molaire) (Wandersman & Delepelaire, 2004). Il est complexé avec d'autres molécules, telles que les protoporphyrines (hème) ou des protéines (ferritine, transferrine, lactoferrine, hémoglobine...). La constante d'affinité de ces protéines pour le fer est très élevée (10^{36}) et elles ne sont que partiellement saturées (30 à 40% pour la transferrine sérique humaine) (Griffiths et al, 1988).

3-Système d'acquisition du fer par *S. aureus*

Plus de 95% du fer dans le corps humain se trouve dans les cellules, ce qui pose un problème pour les bactéries comme *S. aureus*. Pour contourner cet obstacle, *S. aureus* a développé des mécanismes de récupération de fer extracellulaire.

Systèmes indirects d'acquisition de fer : En condition de carence en fer, *S. aureus* synthétise des sidérophores qui sont la staphyloferrine A, la staphyloferrine B et les auréochélines lui permettant d'acquérir le fer qui existe dans le milieu extracellulaire (Courcol et al., 1997; Drechsel et al., 1993; Konetschny-Rapp et al., 1990). La staphyloferrine A et la staphyloferrine B appartiennent à la famille des polycarboxylates qui utilisent potentiellement la transferrine comme substrat. Récemment, un nouvel opéron nommé *sbm* a été décrit. Il est composé de neuf gènes qui codent pour la biosynthèse d'un sidérophore (staphylobactine) jouant un rôle important dans la virulence de *S. aureus* au cours de l'infection (Dale et al, 2004). De plus, certaines protéines de l'opéron *sbm* (*Sbn*CEFH) interviennent dans la synthèse de la staphyloferrine B (Cheung et al, 2009) démontrant un lien entre ces 2 sidérophores. Enfin, *S. aureus* peut utiliser des sidérophores produits par d'autres bactéries comme l'entérobactine (sidérophore de la famille des catécholates), le ferrichrome (hydroxamate) et l'aérobactine (hydroxamate) suggérant une potentialisation de la virulence des bactéries (par acquisition accrue de fer) dans les infections polymicrobiennes (Harrison et al, 2008).

Systèmes directs d'acquisition de fer : *S. aureus* est capable d'interagir directement avec les sources de fer présentes chez l'hôte au cours de l'infection. *S. aureus* possède des protéines de surface capables de séquestrer le fer. StbA (staphylococcal transferrin binding protein A) ou IsdA et IsdB ont été décrites comme

pouvant lier la transferrine et l'hème (Mazmanian et al, 2003; Taylor & Heinrichs, 2002 ; Pishchany et al, 2009). Récemment, il a été démontré que *S. aureus* utilisait préférentiellement l'hème comme source de fer au début de la croissance bactérienne et au cours de l'initiation de l'infection (Skaar et al, 2004) grâce à une forte spécificité pour l'hémoglobine humaine (Pishchany et al, 2010). Le système Isd (Iron-regulated surface determinant) est le premier système d'acquisition d'hème identifié chez *S. aureus* (Mazmanian et al, 2002; Mazmanian et al, 2003). Le détail de son fonctionnement est traité ci-après (paragraphe 7).

Récemment, un autre système de transport d'hème, le système Hts, de type ABC transporteur a été identifié. Il est composé d'une lipoprotéine (HtsA) et deux perméases (HtsB et HtsC). Ce système est plus utilisé par *S. aureus in vitro* qu'IsdDEF et il jouerait un rôle important dans la pathogénicité de *S. aureus* chez l'hôte (Skaar et al, 2004). En effet, au cours d'expériences menées *in vivo* chez *C. elegans* et la souris, les mutants des gènes codant pour Hts étaient significativement moins virulents qu'une souche sauvage (*S. aureus* Newman). De plus par marquage des isotopes, Skaar et al. ont montré que l'acquisition du fer lié à l'hème semblait majoritairement utilisée par *S. aureus* au cours des infections par rapport au fer liée à la transferrine. Or un des systèmes de transport préférentiel de l'hème est assuré par les protéines Hts. En conclusion, les systèmes d'acquisitions d'hème Isd et Hts permettent à *S. aureus* d'être saturé par l'hème après 15 min d'incubation avec l'hème exogène (Mazmanian et al., 2003 ; Rouault, 2004).

4-Transport du fer à travers la paroi bactérienne

Le transport du fer du milieu environnant à l'intérieur de la bactérie implique la translocation de molécules à travers l'enveloppe de la paroi bactérienne (diamètre 50-100 nm) et la membrane cytoplasmique de *S. aureus*. Ce transport à travers la membrane se fait en général par l'intermédiaire des systèmes ABC transporteurs. Plusieurs ABC transporteurs ont été identifiés dans le génome de *S. aureus*. Ils sont codés par les opérons *sitABC* (Cockayne et al, 1998), *sstABCD* (Morrissey et al, 2000), *fhuCBD/CBG* (Cabrera et al, 2001; Sebulsky et al., 2000), *fhuD1/ fhuD2* (Sebulsky & Heinrichs, 2001) et *sirABC* (Heinrichs et al., 1999 ; Dale et al., 2004). Les substrats transportés par les systèmes *sst* et *sir* sont inconnus tandis que les systèmes FhuCBD avec *fhuD1* et *fhuD2* sont impliqués dans le transport du complexe Fe³⁺-hydroxamate.

5-Libération du fer dans le cytoplasme

Les monooxygénases de l'hème catalysent la dégradation oxydative de l'hème en biliverdine, CO₂ et fer (Maines, 1997). Cette réaction est conservée entre les mammifères et certaines espèces bactériennes et remplit des fonctions différentes en fonction de l'organisme en question. À ce jour, deux types de systèmes d'oxygénase de l'hème ont été décrits chez les bactéries à Gram positif dont le système IsdG et IsdI chez *S. aureus* (Wilks & Schmitt, 1998; Wilks, 2002; Skaar et al, 2004; Wu et al, 2005).

6-Régulation du métabolisme du fer

La régulation du métabolisme du fer chez les bactéries se réfère à la notion que les bactéries doivent être capables de sentir et de répondre adéquatement à la nécessité d'acquérir du fer à partir d'un environnement 'hostile' (Holmes, 2000 ; Torres et *al*, 2010). Cette réponse intervient au niveau de l'expression des gènes codant pour les facteurs qui sont responsables de cette absorption, et comprend la transcription, la traduction, et les étapes post-traductionnelles de mécanismes de régulation mais également de facteurs de virulence (cytolysine, protéines d'immunomodulation) (Holmes, 2000; Torres et *al*, 2010; Johnson et *al.*, 2011). Dans de nombreuses bactéries pathogènes, cette activité est accomplie par le répresseur global d'absorption du fer (Fur) (Hantke, 1981; Hantke, 2001; Xiong et *al*, 2000; Horsburgh et *al.*, 2001). La protéine Fur se lie à la région promotrice du système d'attaque du ferrichrome (*fhu*), située sur un opéron qui code pour deux perméases du ferrichrome et une protéine liant l'ATP (Xiong et *al*, 2000). De plus, l'inactivation de *fur* et *perR*, un homologue de *fur*, conduit à une réduction de la virulence dans un modèle murin d'infection à *S. aureus* (Horsburgh et *al*, 2001).

7-Fonctionnement du locus *isd*

Le locus *isd* est le système majeur d'acquisition de fer au niveau de la paroi cellulaire chez *S. aureus* (Skaar & Schneewind, 2004). La caractérisation de ce système a fourni de nouvelles avancées dans le fonctionnement de la paroi cellulaire et les interactions hôte-pathogène.

Le locus *isd* est composé de dix gènes exprimés dans les conditions de carence en fer. Ces gènes codent pour quatre protéines de surfaces (IsdA, IsdB, IsdC, IsdH (ou HarA)), un système de transport membranaire (IsdD, IsdE, IsdF), une transpeptidase (SrtB) et deux monooxygénases (IsdG, IsdI) (Figure 7). Excepté la sortase B (SrtB), toutes les protéines du système Isd sont capables de lier l'hème *in vitro*. De plus, IsdB et IsdH sont responsables de l'interaction avec l'hémoglobine et le complexe hémoglobine-haptoglobine respectivement (Dryla et *al*, 2007; Dryla et *al*, 2003; Torres et *al*, 2006 ; Pishchany et *al*, 2009). Les quatre protéines de surface IsdABCH présentent un à trois domaines NEAT responsables de l'interaction avec l'hème ou les hémoprotéines. De plus, IsdA, IsdB et IsdH présentent des motifs LPXTG qui sont ancrés à la surface par l'intermédiaire de la sortase A, tandis que IsdC présente un motif NPQTN ancré par l'intermédiaire de la sortase B (Marraffini & Schneewind, 2005; Mazmanian et *al*, 2002). À l'inverse des protéines ancrées par la sortase A, IsdC est présente dans la muréine de la paroi de *S. aureus* et cette position unique est très importante pour le passage de l'hème à travers la paroi (Mazmanian et *al*, 2003). Sur la base de la localisation potentielle de ces quatre protéines de surface, un modèle pour le transport de l'hème par le système Isd a été proposé. Après la liaison des hémoprotéines aux protéines de surface IsdB et IsdH, l'hème est prélevé par IsdA suivant un mécanisme inconnu jusqu'à présent. L'hème est ensuite transféré directement de IsdA à IsdC à travers la paroi, et transporté à travers la membrane cytoplasmique par l'intermédiaire de

l'ABC transporteur IsdDEF (Liu et *al.*, 2008; Muryoi et *al.*, 2008; Pluym et *al.*, 2007). Une fois dans le cytoplasme l'hème est dégradé par les monooxygénases IsdG et IsdI et le fer est ainsi libéré pour être utilisé par la bactérie (Skaar et *al.*, 2004).

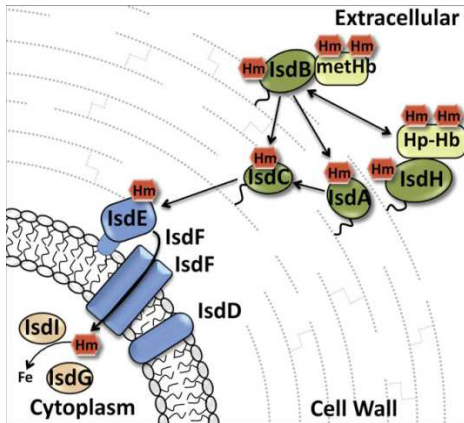


Figure 7 : Représentation schématique des composants du système de transport de l'hème Isd (Grigg et *al.*, 2010).

Le transport et la libération du fer fixé à l'hème sont réalisés par l'action coordonnée de 9 protéines Isd. IsdA, IsdB, IsdC, et IsdH (vert) sont ancrées de façon covalente à la paroi bactérienne. IsdE et IsdF (bleu) sont les protéines de liaison et des perméases constituant un ABC transporteur. IsdE est représenté en liaison avec l'hème avant de former un complexe avec IsdF pour le transport transmembranaire de l'hème. IsdD (bleu) est une protéine membranaire dont la fonction est inconnue. IsdG et IsdI sont des monooxygénases cytoplasmiques dégradant l'hème et libérant le fer. Les ligands préférentiels pour les protéines membranaires sont représentés comme l'hème (Hm), la méthémoglobine (metHb), l'haptoglobine (Hp) et l'hémoglobine (Hb). Par souci de simplicité, IsdB et IsdH sont indiqués en interaction avec un seul ligand protéique, mais IsdB se lie également avec Hp-Hb et IsdH avec la metHb. La voie préférentielle du transfert de l'hème à travers la paroi bactérienne est représentée par des flèches.

F) *Staphylococcus aureus* : agent colonisant et agent infectant

Comme cela a déjà été souligné, toutes les plaies chroniques sont colonisées par des bactéries de la microflore commensale cutanée. Par conséquent, la prise en charge des plaies du pied chez le patient diabétique nécessite de faire la distinction entre une plaie colonisée et une plaie infectée et en particulier entre bactéries colonisantes et bactéries infectantes.

Cependant, aucun outil validé ne permet de distinguer les plaies colonisées des plaies infectées. L'étude du potentiel de virulence des bactéries impliquées pourrait être la clé pour permettre cette distinction.

Plusieurs études axées sur les infections des plaies du pied à *S. aureus* chez des diabétiques ont été menées à l'INSERM U1047 (UFR de Médecine Montpellier-Nîmes, site de Nîmes).

Une première étude, réalisée en 2007, suggérait l'intérêt de l'étude des gènes de virulence par biopuce à ADN pour la distinction entre infection et colonisation et la coexistence probable de deux populations de *S. aureus* sur les plaies chroniques : une population de souches « colonisantes » et une population de souches « infectantes » (Sotto et *al*, 2007). La puce à ADN utilisée contenait 33 cibles : des gènes codant pour des facteurs de virulence et de résistance spécifiques du *S. aureus*. Grâce à cette étude, les auteurs ont démontré que les souches de *S. aureus* isolées des plaies de grade 1 (non infectées selon la classification de l'IWGDF) avaient un faible potentiel de virulence (aucun gène de virulence présent) comparativement aux souches isolées de plaies de grade 2 à 4.

En 2008, une deuxième étude multicentrique, nationale avait pour but d'évaluer le potentiel de virulence de 132 souches de *S. aureus* isolées au cours de plaies chroniques du pied diabétique (Sotto et *al*, 2007). Cette nouvelle étude avait deux objectifs. Le premier concernait l'identification de marqueurs génétiques permettant la distinction entre plaie colonisée et plaie infectée. Le deuxième objectif correspondait à l'identification de marqueurs génétiques qui auraient une valeur pronostique dans le devenir des plaies. Pour cela, les auteurs ont étudié la présence ou l'absence de 31 des principaux gènes de virulence connus chez *S. aureus* par PCR, évalué la clonalité de ces souches (PFGE, spa-typing, SCCmec) et étudié leur potentiel de virulence sur un modèle *in vivo* : le nématode *Caenorhabditis elegans*. Une combinaison de gènes de virulence obtenue à partir d'un modèle de régression logistique et des courbes ROC a retenu 5 gènes : *cap8*, *sea*, *sei*, *lukDE* et *hlgv*. Ces gènes permettraient de distinguer les plaies non infectées (grade 1) des plaies infectées (grades 2-4) et de prédire l'évolution de la plaie lors du suivi clinique. L'étude du potentiel de virulence par *C. elegans* a confirmé la coexistence de ces 2 populations de *S. aureus* présentant une virulence très différente et ce quel que soit leur résistance.

Les objectifs de cette Thèse étaient :

- d'identifier des marqueurs génétiques de *S. aureus* permettant de distinguer les plaies colonisées des plaies infectées,
- d'identifier des clones de *S. aureus* permettant cette distinction,
- d'identifier des clones de *S. aureus* permettant de distinguer des plaies infectées profondes de plaies compliquées d'ostéites.

Travail n°1

New microbiological technologies to manage patients with diabetic foot infection

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New microbiological technologies to manage patients with diabetic foot infection

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Short running title: Diabetic foot and new techniques

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Abbreviations: DFI, diabetic foot infection; IWGDF, International Working Group on the Diabetic Foot; SPILF, French Society for Infectious Diseases (*Société de Pathologie Infectieuse de Langue Française*).

1. Introduction

Foot ulcers are common in diabetic patients, with a prevalence as high as 25% [1]. Diabetic foot infection (DFI) is a frequent (40-80%) and costly [2]. The complications of these ulcers represent a major cause of morbidity and mortality. DFI is estimated to be the most common cause of diabetes-related admission to hospital and remains one of the major pathways to lower-limb amputation [3-5]. The risk of hospitalization and lower-extremity amputation was ≈ 56 and 155 times greater for diabetic people who had a foot infection than for those without, respectively [6]. In the Eurodiale study, 58% of 1,229 diabetic patients consecutively attending one of 14 foot clinics in ten European countries with a new foot ulcer had a wound which was clinically infected [7]. In our own experience, prognosis of DFI remains poor as nearly half of patients admitted in specialized French foot clinics for DFI had some forms of lower-limb amputation and 23 of the 291 patients included into this study died during the one-year study period [8].

The clinical diagnosis of infection may, however, be made difficult. Indeed, the presence of peripheral arterial disease, neuropathy or impaired leukocyte functions may reduce the local inflammatory response and classical signs or symptoms of local infection [9,10]. Moreover, systemic signs of toxicity, such as leukocytosis or fever, may be lacking or appear late, even in severe cases [11-13]. Thus, neither local nor systemic inflammatory signs or symptoms and biological markers should be regarded as reliable for diagnosing foot infection in diabetic individuals. Other aspects of DFI are still open to debate: how to differentiate infection from colonization, when to use antibiotic treatment and for how long? How to manage osteomyelitis? Recently different bacteriological laboratory interventions that decrease reporting time have been developed. These promising new broad-based techniques have merit, based on their ability to identify rapidly a lot of bacteria, virulence and resistance profiles, organisms difficult to culture or newly emerging strains, as well as their capacity to track disease transmission.

2. Differentiation between infection/colonisation

2.1 Clinical aspects

As all chronic wounds are colonized by microorganisms, the diagnosis of DFI should not be based on the microbiological analysis of a wound culture but on clinical findings [5,14,15]. The International Working Group on the Diabetic Foot (IWGDF) and the Infectious Diseases Society of America (IDSA) have

developed clinical criteria for recognizing and classifying the severity of DFI [3,16]. These criteria have been endorsed by the French-speaking Society of Infectious Diseases [14]. Briefly, DFI is defined by the presence at least of two inflammatory manifestations (purulence, or erythema, pain, tenderness, warmth, or induration) and divided in three grades of severity from mild (grade 2) to severe (grade 4) infection, according to the extent of tissue involvement and presence of systemic toxicity or metabolic derangement. This classification system has been validated by a prospective study [17]. Nevertheless, as mentioned above, clinical signs or symptoms of foot wound infection may be much reduced in diabetic patients. This means that reliance on these signs for diagnosis may result in some infections being undetected when they first present. The resultant delay in starting treatment may lead to progression of infection from limited to severe and limb-threatening. This progression is often made rapid because of associated ischemia, diabetic immunopathy and the particular anatomic characteristics of the foot [18,19]. Even if the diagnosis of grade 3-4 is relatively easy, the distinction between grade 1 (uninfected ulcers) and grade 2 (infected ulcer) is often difficult for a non-trained physician. This favors the emergence of antimicrobial resistance, increases financial costs and may cause adverse events [3].

Various clinical factors have been proposed as being suggestive of the presence of DFI when classical signs are not obvious. These include the identification of friable granulation tissue, delay in healing despite otherwise adequate ulcer management and the occurrence of an unexplained hyperglycemia [20-22]. From a practical point of view, however, such signs are either too subtle or too non-specific for general use. Lipsky et al. developed a 10-item DFI wound score incorporating semi-quantitative grading of both wound measurements and various infection parameters. This score could appear to be a reliable and useful tool for predicting clinical outcomes [23].

Recently new technologies have been developed and could help clinicians in this differentiation between infection and colonisation.

2.2 Use of microarrays: Assessing bacterial virulence and resistance

Identification of the virulence power of bacteria might be of value in the future routine diagnosis of infection. New technologies such as DNA micro-array and multiplex real-time PCR offer a unique

opportunity to analyze both the virulence and resistance potential of microorganisms. This method of miniaturized genotyping can rapidly and reliably detect the presence of genes encoding for various virulence and antibiotic resistance factors [24-26]. Using this method, both virulence and resistance genes were far more often present in clinically infected diabetic foot wounds than in uninfected wounds [27]. In cultures from foot wounds from diabetic individuals who had not been exposed to recent antibiotic treatment and were positive only for *S. aureus*, we previously found that virulence factors were present in <10% of isolates from patients with uninfected ulcers but in >98% patients with ulcers that were clinically infected [27]. In addition, the presence of virulence factors at either presentation or at follow-up in patients with clinically uninfected wounds appeared to be predictive of a poor clinical outcome. In another study, we confirmed and extended our previous results using multiplex PCR assays to evaluate 31 of the most prevalent virulence-associated genes in *S. aureus* strains isolated from DFUs [28]. The combination of 5 genes (*sea*, *sei*, *lukDE*, *hlgv* and *cap8*) was the most predictive for differentiating grade 1 (clinically uninfected) from grade 2-4 ulcers, with a sensitivity and a specificity of 0.98 and 0.87, respectively. Recently a new generation of oligonucleotide DNA array has been developed (Figure 1). The *S. aureus* genotyping kit is an array hybridisation kit for the simultaneous DNA-based detection of resistance genes and pathogenicity markers of *S. aureus* and assignment of unknown *S. aureus* isolates to known strains. The results offer more information than traditional tools to study the clonality of bacterial strains such as Pulsed-Field Gel Electrophoresis, or MultiLocus Sequenced Type. The experiment is performed in a strip, which contains 334 probes (in duplicate) printed onto an array located in the bottom of this strip. A reader can scan and analyse at the same time all the data. The presence of a gene is detected by the presence of two black points. The absence of a gene is detected by the absence of the points.

Using this method we confirmed the existence of two populations of *S. aureus* in a national prospective study [29]. We determined that colonizing *S. aureus* strains (Grade 1) with a favourable outcome belonged to same clonal complexes CC5/CC8 ($p<0.001$). If we used the colonizing CCs to differentiate uninfected from infected ulcers, sensitivity was 0.59 and specificity 0.95 and positive and negative predictive values were 0.88 and 0.79, respectively. Moreover, refined analysis of virulence factors associated with infecting strains, using DNA array technology, identified two highly prevalent factors *lukDE* and *hlgv*, as suitable genetic markers to differentiate colonizing from infecting *S. aureus* strains ($p<0.005$). From the logistic model

analysis, *lukDE* was the most predictive gene for the favorable outcome of uninfected DFU: sensitivity was 0.965 and specificity 0.667, and positive and negative predictive values were 0.902 and 0.857, respectively [29]. Another gene marker was identified: *edin* genes that encode for the epidermal cell differentiation inhibitors, a group of toxins targeting RhoA master regulator of the actin cytoskeleton. This group of genes was associated with moderate-to-severe infections and represents a bonafide subsidiary predictive risk marker of DFI [30].

For discriminating infected from uninfected diabetic foot ulceration due to *S. aureus*, the determination of clonal complexes or some virulence-associated genes may be of practical value, especially as the method we used is relatively easy to perform, has low cost (~US\$5 (PCR) to 60 (array) per assay) and gives rapidly available results. The observation of the coexistence of two *S. aureus* populations (a colonizing versus and an infecting one) in DFI, whatever they were or not methicillin-resistant, is an important way for diagnosis. Our different results also confirm the importance of a good microbiological sampling method including the debridement of the wound as debridement decreases the bacterial load and contributes to remove the colonizing bacteria. Analysis of virulence potential (by genotyping methods) would be of value for the diagnosis of diabetic foot wounds in routine clinical practice.

3. Antibiotic treatment in DFU

3.1 Interest of new technologies in DFU

The increasing prevalence of antibiotic-resistant bacteria in DFUs, particularly methicillin-resistant *S. aureus* (MRSA), both as colonizers or pathogens [31-33], is problematic. MRSA requires targeted antibiotic treatment and its involvement is generally considered to be associated with a poor outcome [34,35]. In a prospective study we recently conducted, *S. aureus* was the most common pathogen isolated, accounting for 36.5% of all isolates; and 37.4% of these were MRSA. Overall, MRSA was isolated in 37 of the 188 (19.7%) patients included in the study [36]. Using multivariate analysis, however, we found that the presence of MRSA did not appear to have a significant effect on wound healing time, confirming some previously published studies [37-39]. Although diabetic foot clinics are considered as a major reservoir for MRSA, a recent study suggests that they did not play a key role in transmission and spread of MRSA [40].

Potential sources of acquisition of MRSA to patients may include prior hospitalizations and the nurses who provide care at home for dressing changes. There is, therefore, no evidence that colonization by MRSA requires specific intervention, even though infection with MRSA requires early aggressive treatment. Such treatment should be based on empiric broad-spectrum antibiotic treatment (including agents active against MRSA) and this should be later adjusted according to microbiological isolates, *in vitro* sensitivities and the clinical response to empiric therapy. Typically, the only general direction for antibiotic therapy was provided by a microscopic Gram stain category (Gram-positive bacteria, Gram-negative bacteria, or fungi). However the correlation between Gram stain and bacterial cultures are poor in DFI. Definitive results that eliminate the need for broad-spectrum therapy and enable de-escalation and the tailoring of treatment to the most effective antibiotic regimen often require between 2 to 5 days. This gap has been implicated as one reason for high mortality and the emergence of drug-resistant microbes. The gap is even more prolonged for organisms that are fastidious, slow growing, noncultivable, or present as part of polymicrobial infections (due to biofilm organisation of the bacteria). There is evidence that laboratory interventions that decrease reporting time can be effective mainly due to the absence of new antimicrobial agents and the increasing of multidrug resistance bacteria [41].

3.2 New solutions in rapid bacterial identification and resistance phenotype

Several technological advances using simple molecular methods have become available in recent years and show potential for cost benefit, specifically, peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) [42-46] and targeted real-time PCR such as GeneXpert assays [47,48]. PNA-FISH has been the technology most studied in terms of the collaboration between pharmacy and medical intervention. Both PNA-FISH and real-time PCR methods are easy to perform and can be readily adapted into the laboratory workflow. For example, a real-time PCR method is available for rapid testing of presence of MRSA in blood culture bottles or skin and soft tissue. The benefit of this technology was assessed, and clinical improvements, such as decreased length of stay and health care costs, were documented [49-51]. However these technologies had some limitations because of the limited menu of test organisms and the cost. The technologies would benefit from the addition of a multiple-target approach.

There are thousands of pathogens known to cause disease in humans, and standard PCR assays have

been developed to detect the most commonly occurring pathogens. To meet the analytical needs of broad-range microbial identification, two additional strategies are currently in development for clinical laboratories: DGGE, pyrosequencing and multitarget PCRs.

- DGGE: This technique is based on 16rDNA PCR (a universal PCR enables to amplify all the bacteria) and an electrophoresis of the resulting amplicons in a denaturated gradient gel. In a pilot study, denaturing gradient gel electrophoresis led to the identification of more bacteria than did conventional cultures (mean: 2.35 vs 0.8, respectively). DGGE detects pathogenic species not isolated by cultures and can identify significantly more pathogenic species in deep levels compared to superficial levels. This technique proves the need to perform tissue biopsy sampling. However even id DGGE is achievable in 48h, this is time-consuming to perform in routine practice [52].

- Pyrosequencing methods, while accurate [53,54], make large demands on the technician's time to assess and compare sequences. The cost of the instrument is relatively high, but utility has been documented [55]. The technology's use has been mostly limited to identification of microbial isolates and research for the identification of pathogens in chronic wounds [56]. From pyrosequencing data, the notion of pathogroupes (or FEP, functionally equivalent pathogroups) was proposed by Dowd et al. [56]. According to this thesis, certain bacterial species, when they are alone, are considered non-pathogenic or not being able to maintain a chronic infection, may be organized in a biofilm disease and thus participate in the establishment of chronic lesion. Preliminary data of this concept have been published showing the existence of different pathogroupes in chronic wounds. In addition, a nonrandomized distribution exists in the flora of these wounds. For example, a quantitative analysis of the distance between the aggregated bacteria and the surface of the wound showed that *P. aeruginosa* is more localized in the depth of wound in comparison with *S. aureus* and these bacteria are never co-aggregated [57]. This particular distribution of these two bacteria could explain the under-representation of *P. aeruginosa* and the over-representation of *S. aureus* in chronic wounds when conventional cultures of superficial swab samples. Another example, anaerobes are present in most of the FEP and their distribution is again not randomized: aerobic bacteria are more localized on the surface where oxygen is present while anaerobic bacteria are located in niches deprived for oxygen formed by aerobic bacteria.

The concepts of biofilms and pathogroupes could explain the delayed healing, the polymicrobisme, and the low prevalence of anaerobic bacteria in chronic wounds if the collection, transport and culture methods are not optimized. Similarly the existence of new microbial species not yet identified by conventional methods may explain therapeutic failures. New molecular tools have have greatly contributed to our understanding of the role of the cutaneous microbiota in DFU [58].

- New real-time PCR methods specific to syndrome (mainly represented by sepsis) are undergoing evaluation, but concordance with routine methods needs improvement. The Roche Molecular System LightCycler SeptiFast [59] is available and has been evaluated for rapid detection of specific organisms in blood culture bottles. The performance of Septi-Fast for positive blood cultures is evolving. The concordance of SeptiFast and BC for both positive and negative samples was only 86.0% [60], and to date no clinical utility studies have been published. Recently, Sepsitest from Molzym was evaluated. The Sepsitest uses a universal PCR from the 16S rRNA gene, with subsequent identification of bacteria from positive samples by sequence analysis of amplicons. Compared to blood culture (BC), the diagnostic sensitivity and specificity of the PCR were 87.0 and 85.8%, respectively ($n = 342$) [60].

The Prove it sepsis assay (Mobidiag, Helsinki, Finland) is a new DNA-based microarray platform that enables rapid detection and species identification of many pathogens, including bacteria. This technology identifies more than 50 species of gram-positive and gram-negative bacteria that cause most cases of sepsis [61]. It combines genome amplification via traditional culture with PCR and microarray technology for simultaneous detection of many bacterial targets and then enables to determine antibiotic susceptibility profiles of each common bacterial species making easier the antibiotic therapy of patients with sepsis [61].

In this method, DNA was extracted from sample of blood-culture by the use of an automated platform, and proprietary gene regions of topoisomerase genes and the *mecA* gene were amplified by PCR. The PCR amplicons were subsequently overlaid onto the Prove-it tube microarray in which hybridisation was detected in one reaction. Final bacterial identification was by solid-state hardware, with a simple solid-state detector connected to and powered by a computer's USB socket, taking less than 1 min. Interpretation and reporting of results was via Prove-it advisor software, yielding the name of detected microbes to their species, taxon, or both. The software also showed whether the *mecA* gene was present.

Tissari et al. assessed sensitivity, specificity, and turnaround time of this new molecular sepsis assay and compared it with the gold standard, conventional culture-based method. They measured turnaround times for identification of bacterial species for both methods by measuring the time taken from the start of processing a sample to availability of a result from either method to the physician in charge and turnaround times for the sepsis assay were automatically calculated. 3318 blood samples obtained from patients with suspected sepsis were analyzed. After positive blood cultures were identified, the Prove-it sepsis assay was used to establish the bacterial species and putative meticillin-resistance and compared with the results from conventional culture-based bacteriological methods [61]. All positive samples were gram stained and, when necessary, acridine-orange stained. Biochemical tests for bacterial identification and antibiotic susceptibility tests were undertaken. The results show that from the 2107 (86%) positive blood-culture samples 1807 samples included a pathogen covered by the assay. The Prove it sepsis had a clinical sensitivity of 94.7% and a specificity of 98.8%, and 100% for both measures for meticillin-resistant *S. aureus* bacteraemia. The assay was a mean 18 h faster than was the conventional culture-based method, which takes an additional 1–2 working days, thus it could enable fast and earlier evidence-based management for clinical sepsis [61]. One limitation of this study was to be linked to the previous bacterial growth in traditional blood-culture systems. Another major limitation of this system is the high cost of this technology that does not allow for its routine use for bacterial identification from blood.

Finally promising new broad-based techniques have merit, based on their ability to identify organisms difficult to culture or newly emerging strains, as well as their capacity to track disease transmission. Two techniques revolve around mass spectrometry (MS) technology: PCR combined with electrospray ionization-mass spectrometry (PCR/ESI-MS) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS is a new and exciting method to identify bacteria. The advantage of this technology is its high accuracy, its requirement of only the medium to culture the organism, the low consumable cost of which is negligible in comparison to all the classical techniques used in lab and speed of analysis. MALDI-TOF MS relies on identification of protein profiles derived from highly conserved proteins and currently identified microbes, bacteria, fungi, and mycobacteria, to the species level. For MALDI-TOF, the protein profiles are generated from direct ionization of an intact colony or a bacterial protein extract after manual extraction. Identification occurs after a protein's spectral

signature is correlated to a database of spectra collected from reference strains. Recent evaluations showed promise for the identification of microorganisms using MALDI-TOF MS without subculture [62-65]. This rapid bacterial identification just after sample represents a very interesting way to a best use of antibiotics. Moreover a recent publication identified some resistance mechanism by this technology increasing the potential of this technology [66]. The other technology, PCR/ESI-MS, requires DNA extraction reagents used for PCR, such as buffers, enzymes, and primers, which result in higher cost per sample. PCR/ESI-MS instruments measure the mass/charge ratio (m/z) of amplicons, generated by multiplex PCRs that target several loci within bacterial or fungal genomes. The method targets both conserved and species-specific genetic regions to identify microbes based on amplicon base compositions relative to a known database of microorganisms [67]. Using the Abbott PLEX-ID or the Ibis T5000 instruments, PCR/ESI-MS is known for its ability to directly detect and identify bacteria and associated antibiotic resistance genes, viruses, fungi, and mycobacteria from clinical specimens and from isolates. Most recently, the method produced highly accurate results when used to identify pathogens directly from blood culture bottles [68]. Genetic identification allows for discrimination to the strain type and allows identification of antimicrobial resistance genes. This technology could have an interesting future because it could be used directly from the clinical specimen. PCR/ESI-MS requires approximately 4 to 6 h for specimen testing. To date except the cost, the main limitation of this technology is that some errors in interpretation can occur when combinations of staphylococci and streptococci or of staphylococci and enterococci are found in the same sample, a frequent situation in DFU [68]. Moreover misidentifications also occur when large differences in the bacterial densities of microbes are observed for mixed infections, such that only one of the two organisms is detected.

4. Diagnostic of osteomyelitis

4.1 Interest in DFU

Dealing with osteomyelitis can be difficult in the management of diabetic foot infection because of the presence of many controversial aspects such as the definition of various terms, diagnostic strategy and therapeutic options. A consensual guideline has been recently published. To manage this pathology, it is now well established that bone biopsy is the gold standard test. This technique is useful for reliably recovering the

pathogens responsible for bone infection and for determining their profile of susceptibility to antimicrobial agents. One of the main limitations of this technique is the time to obtain results (approx. 2 weeks). Moreover classically bacterial documentation was not reliable, as it was based on the results of superficial sample cultures; in most of the studies, bone biopsy was not performed. When the results of bone-biopsy cultures are reported, they usually referred to deep-tissue samples taken during debridement rather than true biopsies. The antimicrobial agents usually administered to patients treated medically for foot osteomyelitis were fluoroquinolones, clindamycin and rifampicin, agents that reach the highest concentrations in bone, and maintain activity against surface adhering, slow-growing, and biofilm-producing microorganisms. These antimicrobial agents also have the highest risk of resistant-mutant selection and should, therefore, only be administered in effective combinations (where both agents are active against the pathogens) to prevent the emergence of resistant bacteria (especially with rifampicin) [69]. To ensure that drug combinations are fully active against pathogen(s) involved in bone infection, the choice of agents should ideally be based on the results of bone cultures. Found a new technology that provides a result in one day would be helpful.

4.2 Arrays

4.2.1 GeneXpert

Several studies have evaluated the role of PCR in the diagnosis of bone and joint infections. However, most of the PCR methods used have limitations, such as complex technical requirements, extended hands-on time and test turnaround time, and poor specificity and sensitivity, which represent barriers to routine use [70]. Furthermore, to date, none of these assays has been able to detect antibiotic resistance at the same time.

Recently, the Xpert MRSA/SA SSTI real-time PCR assay on GeneXpert has become commercially available and has been FDA cleared and EC (European Community) marked for the detection of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in skin and soft tissue infections [49].

The GeneXpert MRSA/SA SSTI is an automated closed system that integrates the purification of the sample, amplification of nucleic acid and detection of the target sequence by real-time PCR. The extraction, amplification, and detection steps take place in different chambers of a self-contained, single-use cartridge containing all the reagents necessary for the detection of the bacterial targets [51]. The primers and probes

detect sequences within the staphylococcal protein A (*spa*) gene, the gene for methicillin resistance (*mecA*), and the staphylococcal cassette chromosome (SCCmec) inserted into the *S. aureus* chromosomal *attB* insertion site, as well as an internal-control sample processing control (SPC) (*Bacillus globigii*). The overall process is complete in about an hour and the expected results are as follows: methicillin-resistant SA (MRSA) positive / positive SA (MRSA target sequences are detected and SA), MRSA negative / positive SA (only the target sequence of SA is detected) and MRSA negative / HER negative (the target DNA sequence of SA is not detected). Although the manufacturer does not recommend it, in case of negative MRSA / SA negative, the result was extrapolated SCN resistant to methicillin (SCNMR) (if *mecA* positive) or SCN susceptible to methicillin (SCNMS) (if *mecA* negative) [49].

In practice, PCR results should be communicated to clinicians as quickly as possible to optimize antibiotic therapy at the earliest. The real-time PCR Xpert MRSA/SA SSTI can meet this expectation, since this test is easy to use, does not require a seasoned staff to molecular biology techniques, rapid (results in about an hour) and can be done demand. The relevance of this test is twofold, as it allows one hand, to increase the adaptation of antibiotic therapy and, secondly, to help clinicians to stop or not to initiate antibiotic therapy for bacteremia with *S. aureus*. Like any technological improvement, the impact thereof cannot be ensured unless there is coordination between the laboratory, the referent infectious and clinical services [51,70].

Dubouix-Bourandy et al. evaluated the Xpert MRSA/SA SSTI directly on perioperative bone and joint samples. 135 samples from 105 patients were analyzed using this technology and the results show that the sensitivity and specificity for detection of methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and methicillin-resistant coagulase-negative *Staphylococcus* were, respectively, 100% and 98.3%, 100% and 100%, and 100% and 95.3%. The median total test turnaround time was about 72 min for PCR versus 79 h for culture. Using these rapid results, appropriate antibiotic treatment could be rapidly initiated [49].

4.2.2 Prove it Bone and Joint

The Prove-it Bone and Joint assay (Mobidiag, Helsinki, Finland) is a new DNA-based microarray platform for bone and joint infections. This technology allows the identification of over 60 bacteria and *mecA* resistance marker directly on clinical sample and without bacterial culture prior by providing

accurate DNA-based information [61]. It is based on the extraction of bacterial DNA directly from a joint and joint sample (bone biopsy, tissue or synovial fluid) and combine genome amplification via traditional culture with PCR and microarray technology. The simultaneous detection of many bacterial targets and the determination of antibiotic susceptibility profiles of each common bacterial species make easier the antibiotic therapy of patients with sepsis. A Prove it software provides automated detection, analysis and result reporting [61]. The assay time was about 3.5 h without the DNA extraction time needed. The Prove it Joint and Bone is currently available for research application use only because of the cost of this new technology.

4.2.3 Future approach

In the future, new approaches could be a useful tool to document osteitis in DFU. Recently Valour et al. have observed that delta-toxin production deficiency in *S. aureus* could be a diagnostic marker of bone and joint infection chronicity [71]. This approach must be validated in DFI condition but this needs exclusively the detection of lack of this toxin (peak at 3005 ± 5 Thomson) and its allelic variant (peak at 3015 ± 5 Thomson) using MALDI ToF technology in 30 sec after strain culture. Moreover technologies detecting the most prevalent pathogen isolated in a syndrome begin to appear. A panel targeting bone and joint bacteria and the main mechanism of resistance could represent the future in microbiology.

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Conflict of interest

No conflict of interest

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Legends of tables and figures

Figure

Fig. 1. Example of scanning results of *S. aureus* strain.

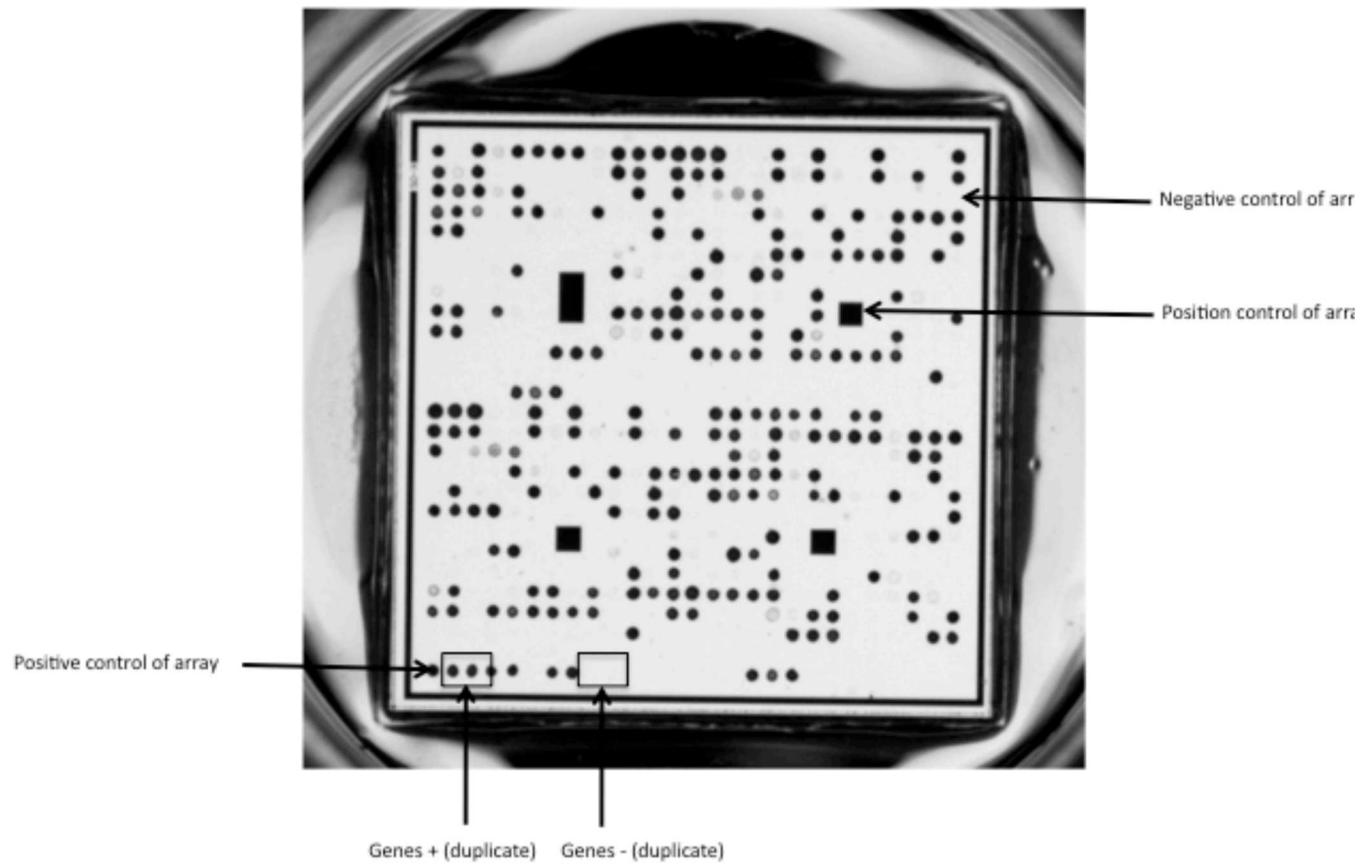


Table 1. Main technologies described in this review with the cost / rapidity of each technique as well as the main limitations.

Technologies	Cost	Rapidity	Limitations
DNA Microarray	++	+	Results depend on a good microbiological sampling
PNA Fish	+	+	Limited spectrum of strains that can be tested
GeneXpert real-time PCR	+	+++	Limited to <i>Staphylococcus</i> sp.
DGGE	+	-	Time-consuming
Pyrosequencing	+++	-	Time-consuming
PCR ESI/MS	+++	-	Misidentifications and misinterpretations. Not a routine solution
MALDI-TOF	-	+++	Need to obtain culture strain

Travail n°2

Distinguishing colonization from infection with *Staphylococcus aureus* in diabetic foot ulcers with miniaturized oligonucleotide arrays: a French multicenter study.

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Les 2 premières études menées par notre équipe avaient démontré que les souches de *S. aureus* isolées des plaies de Grade 1 avaient un faible potentiel de virulence comparativement aux souches isolées de plaies de grade 2 à 4 (Sotto et al, 2007) et qu'une combinaison de 5 gènes de virulence (*sea*, *sei*, *lukDE*, *hlgv* et *cap8*) présents/absents chez *S. aureus* permettait de différencier les plaies colonisées des plaies infectées sur les pieds diabétiques (Sotto et al, 2008). Parmi ces gènes, *lukDE* permettait de prédire la bonne évolution d'une plaie colonisée. L'ensemble de ces travaux avait été mené dans des études monocentriques au sein du CHU de Nîmes.

Pour mieux évaluer l'impact de ces résultats et évaluer la présence des clones de *S. aureus* circulants, une étude multicentrique a alors été menée. Des patients adultes diabétiques de type 1 et 2, ayant une plaie du pied (Grades 1-4) ont été inclus du 1/04/2008 au 30/06/2010 dans 14 Centres Hospitaliers en France (AP-HP Lariboisière, AP-HP La Pitié Salpêtrière, Bordeaux, Clermont-Ferrand, Montpellier, Lille, Narbonne, Nantes, Nice, Nîmes, Perpignan, Reims, St Etienne, Toulouse). Après préparation de la plaie, un prélèvement était réalisé soit par écouvillonnage pour les plaies superficielles, soit par ponction à l'aiguille fine, biopsie tissulaire ou biopsie osseuse pour les plaies profondes. Tous les patients ayant une plaie de grade 1 ont eu une consultation de suivi dans le mois suivant au cours de laquelle un 2^{ème} prélèvement a été effectué. Les prélèvements monomicrobiens à *S. aureus* étaient conservés pour l'étude génotypique par des biopuces à ADN (StaphyType96®, Alere): détection de 330 gènes de virulence/résistance et assignation de l'origine clonale de la souche.

Au cours de cette étude, 195 patients ont été inclus (71% d'hommes, 75 plaies de Grade 1) et 223 souches de *S. aureus* ont été isolées dont 35 SARM lors de la consultation inaugurale ou de suivi.

A l'inclusion, 44 (59%) souches isolées de plaies de Grade 1 appartenaient aux clones CC5/CC8 contre 6 (5%) dans les plaies de Grade 2-4 (p<0,001).

Lors du suivi des patients ayant une plaie de Grade 1 :

i) 57 (76%) ont cicatrisé ou évolué favorablement et 49/57 (86%) présentaient une souche appartenant aux clones CC5/CC8 ;

ii) 18 (24%) se sont aggravées (médiane: 14,5j) et 0/18 présentaient une souche des clones CC5/CC8. Chez tous les patients ayant une plaie qui s'aggrave, la souche identifiée au départ était la même que celle isolée au cours du suivi.

Cette étude a démontré l'existence en France d'une population de *S. aureus* colonisante présente sur les plaies du pied chez le diabétique. L'identification de ces souches permettrait de distinguer les colonisations (Grade 1) des infections (Grade 2-4) et de prédire le devenir de la plaie.

Distinguishing Colonization From Infection With *Staphylococcus aureus* in Diabetic Foot Ulcers With Miniaturized Oligonucleotide Arrays

A French multicenter study

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OBJECTIVE—To extend our previous work on evaluating the use of oligonucleotide arrays to discriminate colonization from infection owing to *Staphylococcus aureus* in diabetic foot ulcers (DFUs).

RESEARCH DESIGN AND METHODS—Patients admitted to 14 French diabetic foot departments for a DFU were screened for entry into the study. At admission, ulcers were classified based on clinical examination according to the Infectious Diseases Society of America system. Only patients with monomicrobial culture for *S. aureus* were included. In persons with an uninfected ulcer, a second wound bacterial specimen was obtained 1 month later. Using oligonucleotide arrays, *S. aureus* resistance and virulence genes were determined, and each isolate was affiliated to a clonal complex (CC).

RESULTS—*S. aureus* was initially isolated from 75 uninfected and 120 infected ulcers; 35 were methicillin resistant. A total of 44 (59%) strains from uninfected DFUs belonged to CC5/CC8 clones vs. 6 (5%) from infected DFUs ($P < 0.001$). During follow-up, 57 (76%) of uninfected DFUs healed or had a favorable outcome; the strain in 49 (86%) of them belonged to CC5/CC8. Conversely, 18 (24%) had a poor outcome but not a single strain belonged to CC5/CC8 clone. Moreover, *lukDE* was significantly associated with a favorable outcome of the wound.

CONCLUSIONS—As suggested by our previous study, the use of DNA arrays appears to be a promising technique that might help distinguishing uninfected from infected wounds, predicting ulcer outcome and then contributing to a more adequate use of antibiotics.

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*A complete list of the members of the French Study Group on the Diabetic Foot can be found in the Supplementary Data.

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Foot ulcers are common in diabetic patients, with prevalence as high as 25% (1). These ulcers frequently become infected, and spread of infection to soft tissue and bony structures is a major causal factor for lower-limb amputation (2), making early diagnosis and adequate treatment essential. As microorganisms are always present on skin wounds, diagnosis of infection must be based not on microbiological findings but on symptoms and clinical signs, as emphasized by the Infectious Diseases Society of America (IDSA), the International Working Group on the Diabetic Foot (IWGDF), and the French Society for Infectious Pathology (3–5). However, owing to the confounding effect of neuropathy and ischemia on local and systemic inflammatory response, diagnosing foot infection at an early stage in diabetic patients is often difficult. Hence, identification of reliable clinical and/or microbiological criteria for diabetic foot infection would be of great value, allowing differentiation of actually infected ulcers from noninfected (colonized) ulcers.

New technologies, such as DNA microarray and multiplex real-time PCR, offer a unique opportunity to rapidly and reliably detect the presence of genes encoding for various virulence factors (e.g., genes encoding the bicomponent toxin Pantone-Valentine leukocidin, *lukF-PV* and *lukS-PV*) and antibiotic resistance factors (e.g., *SCCmec*, the complex mobile genetic elements bringing *mecA*, the methicillin-resistance gene) (6,7). Using this method, we previously found that the combination of five genes (*sea*, *sei*, *lukDE*, *hlgv*, and *cap8*) was highly predictive for differentiating clinically uninfected from infected diabetic foot ulcers (DFUs) (8). Moreover, we showed that the presence of *Staphylococcus aureus* virulence factors, either at presentation or at follow-up in diabetic patients with clinically uninfected wounds, was predictive of a poor clinical outcome (9).

Since then, technological advances have led to the development of a new generation of miniaturized oligonucleotide array to genotype *S. aureus* covering a much larger number of genes. The aim of the current study was to validate such a new tool and to assess its potential advantages over the previous technique to type *S. aureus* isolated from DFUs in several French hospital centers.

RESEARCH DESIGN AND METHODS

Prospective study

We prospectively enrolled a casual sample of outpatients attending 1 of 12 participating French foot clinics (Supplementary Data) between 1 April 2008 and 30 June 2010 for any type of foot ulcer after informed consent was obtained. Patients were included if they had not received any antibiotic agents in the previous week. This study was approved by the local ethics committee (South Mediterranean III) and carried out in accordance with the Declaration of Helsinki as revised in 2000. Every patient was examined by trained physicians to grade infection severity. According to the IDSA/IWGDF criteria (4,5), wounds were considered either uninfected (grade 1) or infected (grade $\geq$ 2).

Study design

After wound debridement, samples for bacterial culture were obtained by swabbing the wound base, needle aspiration, or tissue biopsy and immediately sent to the bacteriology department. Only patients with monomicrobial culture for *S. aureus* were included in the study. Patients with uninfected ulcers did not undergo antibiotic treatment and were monitored closely for 30 days to definitively assess the wound status (infected vs. uninfected). If the wound was considered to be worsening before the follow-up visit, patients were instructed to return to the outpatient facility for early review and a further sample was taken for bacterial culture. At the follow-up visit, if the wound was healing but not completely reepithelialized, a sample for bacterial culture was obtained and the outcome was considered favorable. For completely reepithelialized wounds, no microbiological specimens were sampled and the wound was considered healed.

Microbiological study

Genus, species, and antibiotic susceptibilities were determined using the Vitek 2 card (BioMérieux, Marcy-l'Etoile, France)

and interpreted according to the recommendations of the French Society for Microbiology (10). Susceptibility to methicillin was screened by agar diffusion using cefoxitin disks (BioRad, Marnes-La-Coquette, France) (10).

Oligonucleotide DNA arrays and genotyping

Each *S. aureus* strain collected during the study was analyzed at the INSERM laboratory in Nîmes, France. All the experiments were performed by a PhD student blind to the DFU grade. The Alere StaphyType DNA microarray was used according to protocols and procedures previously described (6,7). The test was able to screen numerous markers simultaneously in 5 h. The DNA microarray covers 334 target sequences including the main virulence and resistance genes. Primer and probe sequences have previously been published (6,7). DNA was extracted from each *S. aureus* strain, and after amplification and hybridization, markers were identified. This technology determines the clonal complex (CC) of strains. A CC may be defined as a cluster of strains (clones) that are close enough together to be claimed to share a common origin. Thus, this group of bacteria is genetically identical to a single ancestral clone. This affiliation of isolates was assessed by an automated comparison of hybridization profiles to a collection of reference strains previously characterized (6,7,11).

In order to determine the CCs of the colonizing strains, we first analyzed the 11 *S. aureus* strains from clinically uninfected ulcers that we isolated in our previous study (8). More information regarding oligonucleotide DNA arrays and genotyping can be found in Supplementary Data.

Statistical analysis

The presence of each gene in *S. aureus* strains was compared as to ulcer grades and outcome of ulcers using Fisher exact test. The most predictive genes for a favorable outcome or healing were expressed by sensitivity, specificity, and positive and negative predictive values; area under the receiver operating characteristic (ROC) curve was calculated by the nonparametric Hanley method. To assess the usefulness of combining several virulence markers, we used logistic regression with a backward procedure to select the most relevant markers; only markers for which area under the ROC curve was >0.80 were initially entered as explanatory variables in the regression analysis.

An ROC curve was then generated for the combination of relevant genes derived from the regression model, and its area was compared with that of every single virulence marker by a nonparametric method adapted to paired data (12). Statistical analysis was performed using the S-Plus 2000 software package (Insightful, Seattle, WA), and results were considered significant for $P < 0.05$.

RESULTS

Clinical and bacteriological data

During the study period, 195 patients were recruited in whom *S. aureus* was the sole organism isolated from the bacterial culture of their wound. Seventy-five wounds (38.5%) were classified as uninfected (grade 1) and 120 as infected (grades 2–4). As shown in Table 1, both groups were well matched for age, sex, type, and complications of diabetes. At the follow-up visit, 24 of initially uninfected DFUs were healed (32%) and 33 had a favorable outcome (44%), whereas 18 (24%) had worsened rapidly ($\leq$ 30 days). No association was found between the uninfected ulcers that worsened and clinical (peripheral arterial disease, neuropathy) or biochemical (HbA_{1c}) parameters. In each case, an *S. aureus* strain was isolated from the second bacterial sampling carried out in those uninfected ulcers that were not completely healed at the follow-up visit, regardless of whether the outcome was favorable. Overall, there were 246 isolates of *S. aureus* (195 at baseline and 51 at the follow-up), of which 40 (16.3%) were methicillin resistant (35 [17.9%] at baseline and 5 [9.8%] during the follow-up period).

CC distribution

CC distribution of isolates allowed comparison between the strains, examination of the biodiversity of the isolates, and determination of their origin and clonality. Using the new technology, the CCs of the 11 colonizing *S. aureus* strains isolated in our previous pilot study (8) were shown to belong only to two clones, CC8–methicillin-sensitive *S. aureus* (MSSA) and CC5–MSSA. Thus, we hypothesized that these two CCs corresponded to colonizing CCs and other CCs were considered infecting CCs.

All the strains from patients included in the current study were analyzed. The results are shown in Fig. 1 and in Supplementary Data. On admission, 44 strains (59%) from clinically uninfected wounds

Table 1—Demographic and clinical characteristics of study patients

Characteristics	Uninfected	Infected	Total	<i>P</i> _{uninfected vs. infected}
<i>n</i>	75	120	195	
Age (years)	66 (41–91)	66.5 (33–101)	66.5 (33–101)	NS
Male/female	51 (68)/24 (32)	88 (73.3)/32 (26.7)	139 (71.3)/56 (28.7)	NS
Type 1/type 2 diabetes (<i>n/n</i>)	12/63	17/103	29/166	NS
HbA _{1c} (%)	8.2	8.4	8.3 ± 2.2	NS
Cardiovascular disease				
Absence	26 (34.7)	28 (23.3)	54 (27.7)	NS
Coronary heart disease	19 (25.3)	44 (36.7)	63 (32.3)	NS
Peripheral arterial disease	42 (56)	80 (66.7)	122 (62.6)	NS
Arterial hypotension	58 (77.3)	96 (80)	154 (79.0)	NS
Stroke	8 (10.7)	8 (6.7)	16 (8.2)	NS
Nephropathy				
Absence	32 (42.7)	37 (30.8)	69 (35.4)	NS
Microalbuminuria	18 (24)	32 (26.7)	50 (25.6)	NS
Proteinuria	17 (22.7)	34 (28.3)	51 (26.2)	NS
Renal failure	22 (29.3)	40 (33.3)	62 (31.8)	NS
Neuropathy				
Peripheral	72 (96)	115 (95.8)	187 (95.9)	NS
Autonomic	14 (18.7)	26 (21.7)	40 (20.5)	NS
Diabetic retinopathy				
Absence	31 (41.3)	48 (40)	79 (40.5)	NS
Nonproliferative diabetic retinopathy	34 (45.3)	57 (47.5)	91 (46.7)	NS
Proliferative diabetic retinopathy	15 (20)	16 (13.5)	31 (15.9)	NS
Lifestyle factors				
Obesity	29 (38.7)	48 (40)	77 (39.5)	NS
Smoking	22 (29.3)	32 (26.7)	54 (27.7)	NS
Alcoholism	8 (10.7)	20 (16.7)	28 (14.4)	NS
Sedentary	54 (72)	93 (77.5)	147 (75.4)	NS
First wound/recurrence	23 (30.7)/52 (69.3)	28 (23.3)/92 (76.7)	51 (26.2)/144 (73.8)	NS
IDSA grade				
1	75 (100)	0 (0)	75 (38.5)	ND
2	0 (0)	38 (31.7)	38 (19.5)	ND
3	0 (0)	70 (58.3)	70 (35.9)	ND
4	0 (0)	12 (10)	12 (6.1)	ND
Samples				
Swabbing	75 (100)	68 (56.6)	143 (73.3)	ND
Needle aspiration	0 (0)	12 (10)	12 (6.2)	ND
Tissue/bone biopsy	0 (0)	40 (33.3)	40 (20.5)	ND
Wound evolution (1 month after enrollment)				
Complete healing	24 (32)			
Improved	33 (44)			
Worsening	18 (24)			

Data are median (25th–75th percentile) or *n* (%) unless otherwise indicated. ND, not determined.

belonged to colonizing CCs compared with only 6 (5%) from infected DFUs ($P < 0.001$). According to the outcome, the prevalence rate of colonizing CCs increased up to 86% if strains isolated from DFUs whose outcome were favorable were also taken into account (Fig. 1 and Table 2). On the other hand, no strains isolated from DFUs with a worsening outcome belonged to colonizing CCs and there was no change of CCs between admission and the follow-up visit. Infecting CCs were recovered from 96%

of *S. aureus* isolated from infected ulcers or with a worsening outcome. Among these infecting CCs, CC45-MSSA was the most commonly identified in 21 strains. If we used the colonizing CCs to differentiate uninfected from infected ulcers, sensitivity was 0.59 and specificity 0.95 and positive and negative predictive values were 0.88 and 0.79, respectively. If colonizing CCs were considered for differentiating DFUs according to the outcome, diagnostic performances were substantially improved (Table 2). Finally,

the methicillin-resistant *S. aureus* (MRSA) strains mainly belonged to CC8-MRSA (Lyon clone) ($n = 25$ strains), the main clone present in French hospitals.

Virulence profile

The univariate analysis found that among the five genes (*sea*, *sei*, *lukDE*, *hlgy*, and *cap8*) that we previously identified as predictive for differentiating infected from non-infected DFUs (8), only two (*lukDE* and *hlgy*) actually distinguished infected from uninfected DFUs ($P < 0.05$) (Table 3).

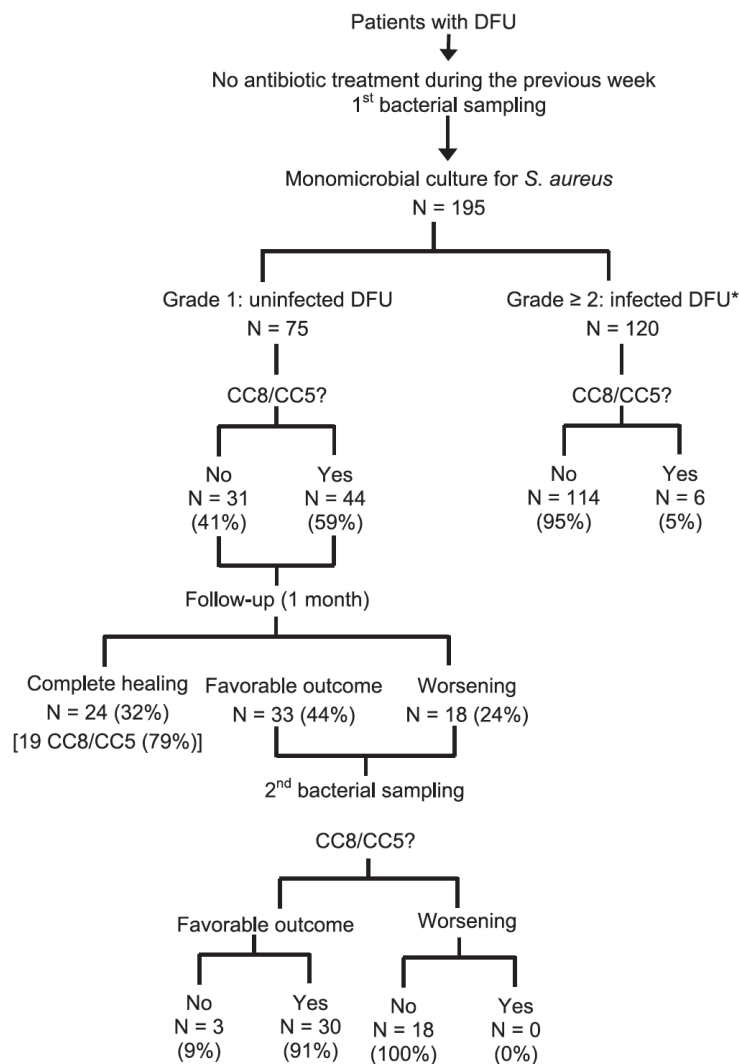


Figure 1—Flow of patients through the study and results of CC8-MSSA/CC5-MSSA obtained of grade 1–4 inclusion and during follow-up of uninfected ulcers. *Grades according to the IDSA/IWGDF classification system (4,5): grade 2, n = 38; grade 3, n = 70; grade 4, n = 12.

However, three of these five genes (*cap8*, *lukDE*, and *hlgv*) differentiated *S. aureus* isolated from uninfected DFUs with favorable outcome and *S. aureus* isolated both from infected and uninfected DFUs with a poor outcome ($P < 0.05$). Moreover, the five genes were significantly associated with the wound outcome: *cap8* and *sei* with a poor outcome as opposed to *sea*, *lukDE*, and *hlgv*, which were associated with DFUs with favorable outcomes (Table 3). In the univariate analysis, 72 additional

genes were found to be significantly associated with the outcome of uninfected ulcers ($P < 0.05$) (data not shown). From the logistic model analysis, *lukDE* was the most predictive gene for the favorable outcome of uninfected DFU: sensitivity was 0.965 (SD 0.025) and specificity 0.667 (0.063), and positive and negative predictive values were 0.902 and 0.857, respectively. Using this model, only two uninfected ulcers were misclassified as they healed despite the absence of *lukDE*; interestingly, one ulcer

Table 2—Distribution of colonizing and infecting clonal complexes of *S. aureus* according to the outcome of DFUs

	CCC	ICC	Total
Healed or with a favorable outcome	49 (86)	8 (14)	57
Infected or worsening	6 (4)	132 (96)	138
Total	55	140	195

Data are n or n (%). Sensitivity, 0.86; specificity, 0.96; positive predictive value, 0.89; negative predictive value, 0.94; accuracy, 0.93. CCC, colonizing CC; ICC, infecting CC.

presented a colonizing CC8 strain and the other a strain positive for Pantone-Valentine leukocidin (PVL). Conversely, *S. aureus* from six ulcers with a poor outcome were positive for *lukDE* gene; however, these strains belonged to infecting CCs.

Genes encoding PVL and exfoliatin A, B, and D toxins were found in 4 (2%), 1 (0.5%), 0 (0%), and 12 (6%) of the isolates, respectively. No differences were found between infected and uninfected ulcers.

CONCLUSIONS—Over the last few years, various DNA array technologies have been developed, but most systems are very expensive, time-consuming, technically demanding, and difficult to adapt to the needs of clinical screening, restricting their use to research laboratories (6,7). In contrast, compared with the first generation of miniaturized oligonucleotide array, the new system we used is more convenient and informative. Each DNA microarray carries a set of 334 different probes (vs. 50 for the first generation), and the use of strip-integrated arrays is time-saving (5 h vs. 1 day for the first generation), easy to perform and to interpret, and allows a large number of samples to be analyzed (96-well strip) (6,7) at low cost (~60 USD, which is three times less expensive than the first generation). Moreover, the new arrays allow for assessment of whether the strains are identical by determining the CCs.

The main result of our multicenter study is that the microarray technology revealed differences between uninfected and infected ulcers and, if the results are confirmed, the use of this technique may enable clinicians to identify infection and to predict the outcome of apparently clinically uninfected wounds. The most interesting contribution of this new microarray is its

Table 3—Main virulence profiles of *S. aureus* isolated from uninfected (grade 1) ulcer at initial sampling and follow-up

	Initial samples		Follow-up			P*
	Uninfected	Infected	Healing†	Favorable outcome	Worsening outcome	
<i>n</i>	75	120	24	33	18	
Markers previously found to discriminate infected/uninfected ulcers						
<i>sea</i>	36 (48)	44 (36.7)	12 (50)	27 (81.8)	6 (33.3)	0.002
<i>lukDE</i>	59 (78.7)	68 (56.7)	22 (91.7)	33 (100)	6 (33.3)	<0.0001
<i>hlgv</i>	62 (82.7)	46 (38.3)	24 (100)	31 (93.9)	9 (50)	0.0001
<i>cap8</i>	25 (33.3)	59 (49.2)	5 (20.8)	4 (12.1)	14 (77.8)	0.0001
<i>sei</i>	29 (38.7)	58 (48.3)	7 (29.1)	5 (15.2)	10 (55.6)	0.02
Other markers found to discriminate infected/uninfected ulcers						
<i>hlyb</i>	54 (72)	53 (44.2)	22 (91.7)	29 (87.9)	6 (33.3)	<0.0001
<i>cap5</i>	51 (68)	62 (51.7)	19 (79.2)	30 (90.9)	5 (27.8)	<0.0001
<i>fib</i>	61 (81.3)	39 (32.5)	23 (95.8)	33 (100)	8 (44.4)	<0.0001
<i>fnbA</i>	34 (45.3)	22 (18.3)	16 (66.7)	25 (75.8)	3 (16.7)	0.006
<i>fnbB</i>	31 (41.3)	18 (15)	15 (62.5)	24 (72.7)	2 (11.1)	0.002
<i>bbp</i>	62 (82.7)	97 (80.8)	19 (79.2)	18 (54.5)	17 (94.4)	0.03
<i>ebpS</i>	45 (60)	32 (26.7)	18 (75)	26 (78.8)	6 (33.3)	0.01
<i>clfB</i>	23 (30.7)	38 (31.7)	17 (70.8)	29 (87.9)	5 (27.8)	0.002
<i>isdA</i>	60 (80)	53 (44.2)	23 (95.8)	32 (97.0)	9 (50)	<0.0001
<i>splA</i>	58 (77.3)	67 (55.8)	22 (91.7)	32 (97.0)	8 (44.4)	<0.0001
<i>splB</i>	55 (73.3)	67 (55.8)	20 (83.3)	32 (97.0)	8 (44.4)	0.0004
<i>cna</i>	21 (28)	55 (45.8)	5 (20.8)	3 (9.1)	10 (55.6)	0.006
Other virulence factors						
<i>lukS-PV lukF-PV</i>	3 (4)	1 (0.8)	1 (4.2)	0 (0)	2 (11.1)	
<i>etA</i>	0 (0)	1 (0.8)	0 (0)	0 (0)	0 (0)	
<i>etB</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
<i>etD</i>	4 (5.3)	8 (6.7)	0 (0)	0 (0)	2 (5.5)	
<i>agrI</i>	54 (72)	72 (60)	18 (75)	27 (81.8)	12 (66.7)	
<i>agrII</i>	13 (17.3)	29 (24.2)	4 (16.7)	4 (12.1)	3 (16.7)	
<i>agrIII</i>	8 (13.3)	18 (15)	2 (8.3)	2 (6.0)	3 (16.7)	
<i>agrIV</i>	0 (0)	1 (0.8)	0 (0)	0 (0)	0 (0)	

Data are *n* (%) unless otherwise indicated. Boldface indicates genes of the uninfected ulcers associated with a worsening outcome. †Results were obtained at baseline.

*Comparison of grade 1 ulcers between those healed or with a favorable outcome at the follow-up and those whose condition worsened.

ability to determine the clonal CCs of the strains. As a result, a lot of CCs were isolated from DFUs (Supplementary Data), demonstrating that a large panel of *S. aureus* is involved in this pathology. The results of this study also clearly suggest that the colonizing *S. aureus* strains belonged to 2 CCs: CC8-MSSA and CC5-MSSA. The distribution of these CCs was significantly different in uninfected and infected DFUs (58 vs. 5%, respectively; $P < 0.001$) and predicted a favorable outcome of the uninfected wounds, as 86% of strains isolated from uninfected ulcers that healed or had a favorable outcome belonged to these CCs. We previously showed that the association of five virulence markers could distinguish uninfected and infected DFUs (8). The current study shows that a single one of the five markers (*lukDE*) may suffice with a high sensitivity (96.5%). The association of CCs and the *lukDE* gene is a powerful

tool for predicting the wound outcome. For example, none of the six ulcers from which *lukDE*-positive *S. aureus* was isolated and that had a poor outcome belonged to a colonizing CC. However, the results of the current study vary from those of our previous study: only two (*lukDE* and *hlgv*) of the five previously identified virulence genes actually allowed the distinction between infected and uninfected DFUs. Moreover, it is worth noting that *cap8* gene was a marker of colonization in our pilot study but a marker of worsening evolution in the current study. Some explanations may be put forward to account for those discrepancies: the current study is multicentric, and the detection tools are different (PCR versus arrays with different targets for the same gene). Moreover, the time between the two studies coincided with a quite marked decrease in the prevalence of MRSA in France (involving

a change in clonal strains). Interestingly, this study showed that all of the uninfected strains that worsened had the same genotype (CC and content of 334 genes), indicating that the wounds were actually infected and not only colonized. These results highlight the potential usefulness of this technique in patients in whom the clinical diagnosis of infection is made difficult by, for example, peripheral arterial disease, neuropathy, or impaired leukocyte functions (13). Additionally, the DNA microarray might provide important help for clinicians and might allow for adequate management of clinically uninfected ulcers carrying *S. aureus*, according to its genotype profile. Results of the current study raise the question about antibiotic treatment in apparently uninfected DFUs and challenge the dogma about abstention of antibiotic therapy in clinically uninfected ulcers (14,15).

Furthermore, this new generation of microarray rapidly gives information about in vitro susceptibility of *S. aureus*, especially about the presence of *mecA* gene, an important therapeutic concern. Worth noting is that the prevalence of MRSA was low in the current study (18%), confirming the decreasing trends in MRSA prevalence in France (16). While 14 patients with uninfected ulcers had an MRSA strain at baseline, only five cases of MRSA were recovered during the follow-up, suggesting that debridement of the wound alone may be sufficient for eradicating MRSA. The favorable outcome associated with uninfected DFU with *S. aureus* belonging to the "infecting" CCI poses a problem. However, this clonal complex does not have to be included in colonizing CC, as three DFUs were actually infected by CCI *S. aureus*.

Finally, an additional valuable feature of the array technology is its ability to identify PVL genes, since these genes coding for a cytotoxin are claimed to be a major threat in severe tissue necrosis (17–21). However, these strains are rarely isolated from chronic wounds (22,23) and their pathogenicity is low in this setting; accordingly, some PVL⁺ strains isolated from grade 1 ulcers were healed 1 month later. This suggests that the portal of entry of PVL⁺ strains is not the chronic wound. The DNA array represents a powerful tool for predicting the evolution of uninfected ulcer, and a clinical study on the cost-effectiveness of this technology on the healing time and amputation rate would be interesting.

One of the main limitations of this study is that it was conducted exclusively on DFUs with *S. aureus* as the sole pathogen, while the flora of DFUs is often polymicrobial. Nevertheless, if we focus on uninfected ulcers, most of them are monomicrobial, and *S. aureus* is one of the most frequently isolated microorganisms. *S. aureus* is also the most common pathogen in infected ulcers, even if the infection is polymicrobial (24). Moreover, to eliminate any influence of another pathogen in the clinical course of the wounds, it was useful to study wounds with only *S. aureus* strains. Hence, this clinical platform appears suitable for use under routine conditions in a microbiology laboratory. Another limitation is that the specimens for culture were not obtained by the same method in all patients. Finally, despite the fact that the clinical diagnosis of infection was made by experts in the field, the interobserver variability in diagnosing infected (vs.

uninfected) DFUs was not tested and is largely unknown.

In conclusion, as our previous study suggested, the miniaturized oligonucleotide arrays are an interesting tool for managing DFUs: they allow an early discrimination between infection and colonization of wounds by *S. aureus*. Some results of this study challenge the current belief that antibiotic therapy is not needed for clinically uninfected DFUs. One concern is that this may encourage clinicians to use this technology for screening apparently uninfected ulcers, contrary to current guidelines.

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A.S. and J.-L.R. researched data and wrote the manuscript. N.Me. researched data. N.Me. performed the statistical analyses. N.J., S.S., A.S., and C.C. researched data and reviewed and edited the manuscript. B.C., L.L., and G.L. researched data and contributed to the discussion. J.-P.L. was involved in the design of the overall study, designed the analysis plan, supervised the analysis, and wrote the manuscript. As the corresponding author and guarantor of this article, J.-P.L. takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript. All the participants of the French Study Group on the Diabetic Foot recruited the patients and reviewed and edited the manuscript.

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Travail n°3

Distribution of *edin* in *Staphylococcus aureus* isolated from diabetic foot ulcers

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L'EDIN (Epidermal Differentiation INhibitor) est un facteur de virulence qui confère des propriétés invasives à *S. aureus*. Trois types d'EDIN ont été identifiés à ce jour : EDIN-A, EDIN-B, et EDIN-C. Ce facteur de virulence représentait une cible intéressante comme outil diagnostique dans les plaies du pied diabétique. Suite aux travaux précédemment menés sur la cohorte française, nous avons voulu évaluer la présence/absence des gènes codant pour l'EDIN sur le panel de *S. aureus* isolées de plaies du pied diabétique en France et évaluer leur rôle comme outil pronostique.

Le panel de souches correspond à celui décrit dans le Travail n°2. Les données cliniques d'évolution des plaies avaient été recueillies prospectivement. La recherche des gènes codant pour les EDIN a été effectuée par biopuce à ADN (StaphyType96®, Alere) pour *edinA* et *edinB* et par une PCR spécifique pour *edinC* (Munro P et al, 2011) du fait des problèmes de détection par la biopuce.

Notre étude a permis d'observer que ce groupe de gènes était significativement associé à des infections modérées à sévères (Grades 3 et 4) et représentait un marqueur prédictif de risque de sévérité pour les plaies à *S. aureus* chez le diabétique.

Distribution of *edin* in *Staphylococcus aureus* isolated from diabetic foot ulcers

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Abstract

Staphylococcus aureus is both a common colonizer of human skin and the most frequently isolated pathogen in diabetes foot infections (DFIs). The spread of DFI to soft tissue and bony structures is a major causal factor for lower-limb amputation. It is therefore of great importance to differentiate colonizing from infecting strains of *S. aureus*. Epidermal cell differentiation inhibitors known as EDIN and EDIN-like factors, a group of toxins targeting RhoA master regulator of the actin cytoskeleton, may confer virulence properties on *S. aureus*. In this study, for the first time, analysis of *S. aureus* strains, recovered in DFIs at an initial stage and during the follow-up, showed that 71.4% of *edin*-positive strains were associated with moderate-to-severe infections (grades 3 and 4 of the IDSA/IWGDF classification) compared with 28.6% of *edin*-positive strains associated with low-grade infections. Most of these strains were *edin-B* positive (86.7%) and belonged to CC25/28-MSSA ($n = 10$). One *edin-B*-positive ST152-MSSA strain was negative for the two highly prevalent predictive markers of infecting strains (*lukDE* and *hlgv*). Collectively, this points towards the *edin-B* encoding gene as a *bonafide* subsidiary predictive risk marker of DFI.

Keywords: Colonization, diabetic foot ulcer, EDIN, oligonucleotide array, severe infection, *Staphylococcus aureus*

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Introduction

Foot ulcers are common in diabetic patients, with a prevalence rate as high as 25% [1]. These ulcers frequently become infected, and spread of infection to soft tissue and bony structures is a major causal factor for lower-limb amputation [2]. *Staphylococcus aureus* is the most frequently isolated pathogen in diabetic foot infections (DFIs). Both the

occurrence and progression of staphylococcal infection result from the action of a variety of virulence factors combined with the immunological status of the host [3, 4]. Bacterial factors are extremely heterogeneous in structure and mode of action, giving the bacteria specific metabolic and adhesive properties, as well as offering them a protection against the large body of innate immune effectors [3]. Defining the contributing role of virulence factors by combining cellular microbiological techniques and epidemiological approaches would be of interest in predicting *S. aureus*-associated risk of peyorative infections [5].

The epidermal differentiation factor (EDIN) and EDIN-like factors of *S. aureus* belong to such factors of well-determined mode of action, although their implication in staphylococcal infection remains ill defined. EDIN belongs to a family of bacterial exotoxins targeting the small host protein RhoA, which they inhibit [6, 7]. This small GTPase is a major

regulator of the host cell actin cytoskeleton [8]. Several cell biology studies have revealed that inhibition of RhoA has a detrimental impact on the cohesion of epithelium and endothelium barriers, which is likely to favour bacterial dissemination [9, 10]. Inhibition of RhoA also impairs complement-mediated phagocytosis [11]. As a whole, a large number of studies aimed at investigating the impact of RhoA inhibition, point towards a contributing role of EDIN factors in bacterial colonization and host tissue invasion [5, 12]. Thus defining the distribution of *edin*-encoding genes in clinical isolates of *S. aureus* might help in predicting the risk factors for severe and spreading infection.

A previous multicentre study conducted on clinical isolates of *S. aureus* had suggested that two clonal complexes (CC) and an array of virulence factors can be used to discriminate uninfected from infected diabetic foot ulcers (DFUs) [13]. Indeed, we determined that colonizing *S. aureus* strains (grade I) with a favourable outcome are linked to the clonal complexes CC5/CC8. In parallel, we defined a group of virulence factors associated with worse outcomes of DFU, notably *sea*, *sei*, *lukDE* and *cap8* [14]. Refined analysis of virulence factors associated with infecting strains, using DNA array technology, identified two highly prevalent factors, *lukDE* and *hlgv*, as suitable genetic markers to predict risk of infection by *S. aureus* ($p < 0.005$). Nevertheless, it would be interesting to determine additional markers, which might be used to discriminate a higher number of colonizing from infecting strains of *S. aureus* in DFU. We therefore analysed the distribution of *edin* genes in clinical isolates of *S. aureus* responsible for diabetic foot infection (DFI) in a French national collection of 195 isolates.

Patients and Methods

Study design

We prospectively enrolled a sample of outpatients attending one of 12 participating French foot clinics between 1 April 2008 and 30 June 2010 for any type of DFU, after informed consent was obtained (Fig. 1). This study was approved by the local ethics committee (South Mediterranean III) and carried out in accordance with the Declaration of Helsinki as revised in 2000. Patients were included if they had not received any antibiotic agents in the previous week. Every patient was examined by trained physicians to grade infection severity. According to the IDSA-IWGDF criteria [15, 16], wounds were considered either uninfected (grade I) or infected (grade ≥ 2). After wound debridement, samples for bacterial culture were obtained by swabbing the wound base, needle aspiration or tissue biopsy and immediately sent to the bacteriology

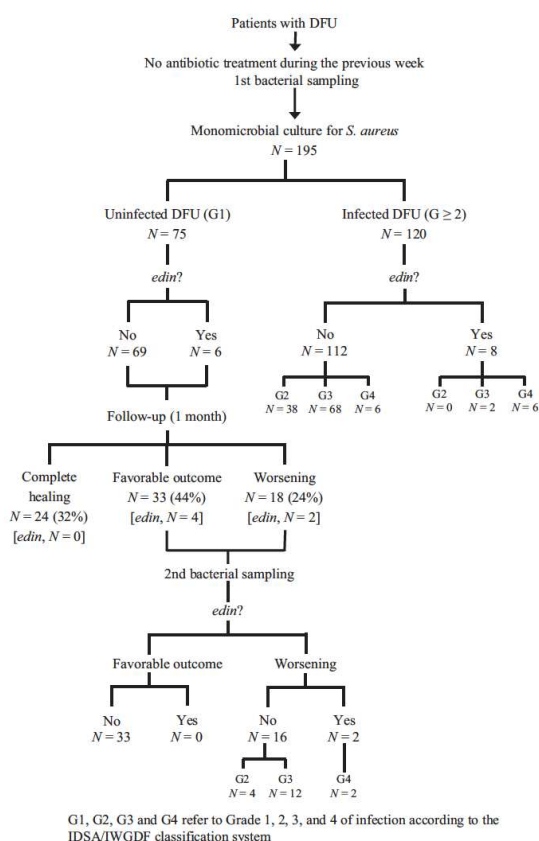


FIG. 1. Flowchart of the study.

department. Only patients with monomicrobial culture for *S. aureus* were included in the study. Antibiotic treatment was not prescribed in patients with uninfected ulcers. Thereafter, patients were closely monitored for 30 days to definitively assess the wound status (infected/uninfected). If the wound condition was worsening before the follow-up visit, patients were instructed to return to the outpatient department for early review and a further sample was taken for bacterial culture. At the follow-up visit, if the wound was healing but not completely re-epithelialized, a sample for bacterial culture was obtained, and the outcome was considered as favourable. For completely re-epithelialized wounds, no microbiological specimens were sampled and the wound was considered healed.

Microbiological study

Genus, species and antibiotic susceptibilities were determined using the Vitek 2 card (BioMérieux, Marcy-l'Etoile, France) and interpreted according to the recommendations of the French Society for Microbiology [17]. Susceptibility to methicillin was screened by agar diffusion using cefoxitin disks (BioRad, Marnes-La-Coquette, France) [17].

Oligonucleotide DNA arrays to determine EDIN-isoforms

Each *S. aureus* strain collected during the study was analysed at the INSERM laboratory in Nîmes, France. The Alere Staphy-Type DNA microarray was used according to protocols and procedures previously detailed [18, 19]. The test was able to screen numerous markers simultaneously in 5 hours. The DNA microarray covers 334 target sequences including two *edin*-isoforms: *edinA* and *edinB*. Primer and probe sequences have been published previously [18, 19]. DNA was extracted from each *S. aureus* strain and after amplification and hybridization, markers were identified. This technology determines the clonal complex (CC) of strains. A CC may be best defined as a cluster of strains (clones) that are close enough together to be claimed to share a common origin. Thus this group of bacteria is genetically identical to a single ancestral clone. This affiliation of isolates was assessed by an automated comparison of hybridization profiles with a collection of reference strains previously characterized [18, 19].

Statistical analysis

The presence of each gene in *S. aureus* strains was compared according to the ulcer grades and the outcome of ulcers using Fisher's exact test. The performances of the *edin*-isoforms for diagnosing grade 4 DFU were assessed as usual. Statistical analysis was performed using the S-PLUS 2000 software package (Insightful Corporation, Seattle, WA, USA) and results were considered significant for $p < 0.05$.

Results

Clinical and bacterial data

During the study period, 195 patients were enrolled. At baseline, seventy-five wounds (38.5%) were classified as uninfected and 120 as infected (Fig. 1). During the follow-up period, 18 patients (24%) returned to the outpatient department because of a rapidly worsening wound: four developed a grade 2 ulcer, 12 a grade 3 ulcer and two a grade 4 ulcer. At the follow-up visit, 33 patients (44%) had their wound not fully re-epithelialized: a sample for bacterial culture was obtained, and the outcome was considered as favourable. In 24 patients (32%), the wound was considered healed.

At baseline, *S. aureus* isolate was found to be positive for *edin* (A and B) genes in 14 patients (7.2%): six isolates were recovered from the patients with clinically uninfected DFUs (8%) and eight from patients with infected DFUs (7%). Six of those 14 patients (43%) had a grade 4 DFI compared with six of the 187 patients (3%) in whom *S. aureus* was *edin*-negative ($p < 0.0003$, Table 1). Other clinical characteristics were

identical for both groups (Table 1). Moreover, no significant differences in infection outcomes were found in the 75 patients of the uninfected group, whether the strains were positive or negative for *edin*.

During the follow-up period, 24 of the initially uninfected wounds completely healed, whereas 33 improved without being closed and 18 worsened. No *S. aureus* strain from the healed wounds was initially positive for *edin* genes. From the six uninfected DFUs with *edin*-positive *S. aureus*, four had a favourable outcome (66.7%), whereas two (33.3%) worsened rapidly (≤ 30 days) with occurrence of a grade 4 DFI. Of note, an *S. aureus* strain was isolated from the second bacterial sampling carried out in those uninfected ulcers that were not completely healed at the follow-up visit, whether the outcome was favourable or not.

Collectively, here we determined that *edin*-positive strains of *S. aureus* were more frequently recovered from severe grade 3/4 ulcers.

Distribution of *edin-A*, *B* encoding genes

The prevalence of *edin*-positive isolates was 7.2% (14/195). DNA array analysis revealed that among the 14 *edin*-positive *S. aureus* strains, only one (0.5%) was *edin-A* positive whereas 13 (6.7%) were *edin-B* positive. The *edin-A*-positive strain of *S. aureus* was recovered from a colonizing sample. The *edin-B*-positive strains were recovered from colonizing samples in five patients and in eight patients suffering from grade 3/4 infected ulcers.

In the second bacterial sampling carried out in those uninfected ulcers that were not completely healed, *edin-B*-positive *S. aureus* was recovered only from the two rapidly and severely worsening DFUs. In the four DFUs with favourable outcome, the strains isolated at follow-up no longer harboured any *edin*-isoforms; moreover, these strains were not the same as initially assessed by difference in their CCs.

If we used the *edin*-isoforms to differentiate localized infections (grade 2–3) from systemic infections (grade 4), sensitivity was 0.5, specificity 0.96, and positive and negative predictive values 0.43 and 0.97, respectively. Moreover, if we used the *edin*-isoforms to predict outcome of initially uninfected DFUs, sensitivity was very poor (11.1%) but specificity high (93.0%); positive and negative predictive values were 33.3% and 76.8%, respectively.

Main characteristics of *edin*+ *S. aureus* isolates

The univariate analysis found that eight genes were significantly associated with *edin*-positive isolates: *lukDE*, *hlgv*, *lukS-PV*, *lukF-PV*, *etD*, *cap5*, *fib*, *splA* and *splB* ($p < 0.05$). In contrast, four genes (*cap8*, *sea*, *cna* and the regulator *agrII*) were significantly associated with *edin*-negative strains ($p < 0.001$).

TABLE 1. Demographic and clinical characteristics of the study population

Characteristics	Patients with <i>edin</i> (+) <i>S. aureus</i> <i>n</i> = 14	Patients with <i>edin</i> (-) <i>S. aureus</i> <i>n</i> = 181	Total <i>n</i> = 195	<i>P</i> <i>edin</i> vs. total
Age (range), y	69 (58–91)	66 (33–101)	66.5 (33–101)	NS
Male/female, <i>n</i> (%)	8 (57.1)/6 (46.9)	131 (72.4)/50 (27.6)	139 (71.3)/56 (28.7)	NS
Type 1/Type 2 diabetes mellitus	3/11	26/155	29/166	NS
HbA1c (%)	8.4 ± 2.9	8.3 ± 2.4	8.3 ± 2.2	NS
Cardiovascular disease				
Absence	5 (35.7)	49 (27.1)	54 (27.7)	NS
Coronary heart disease	6 (42.9)	57 (31.5)	63 (32.3)	NS
Peripheral arterial disease	8 (57.5)	114 (63.0)	122 (62.6)	NS
Arterial hypotension	12 (85.7)	142 (78.5)	154 (79.0)	NS
Stroke	2 (14.4)	14 (7.7)	16 (8.2)	NS
Nephropathy				
Absence	5 (35.7)	64 (35.4)	69 (35.4)	NS
Microalbuminuria	3 (21.4)	47 (26.0)	50 (25.6)	NS
Proteinuria	3 (21.4)	48 (26.5)	51 (26.2)	NS
Renal failure	5 (35.7)	57 (31.5)	62 (31.8)	NS
Neuropathy				
Peripheral	10 (71.4)	177 (97.8)	187 (95.9)	NS
Autonomic	0 (0)	40 (22.1)	40 (20.5)	NS
Retinopathy				
Absence	7 (50)	72 (39.8)	79 (40.5)	NS
Non-proliferative diabetic retinopathy	6 (42.8)	85 (47.0)	91 (46.7)	NS
Proliferative diabetic retinopathy	1 (7.1)	30 (16.6)	31 (15.9)	NS
Lifestyle factors				
Obesity	4 (28.6)	73 (40.3)	77 (39.5)	NS
Smoking	3 (21.4)	51 (28.2)	54 (27.7)	NS
Alcoholism	3 (21.4)	25 (13.8)	28 (14.4)	NS
Sedentary	8 (57.1)	139 (76.8)	147 (75.4)	NS
First wound/recurrence	3 (21.4)/11 (78.6)	48 (26.5)/133 (73.5)	51 (26.2)/144 (73.8)	NS
IDSA grade				
1	6 (42.8)	69 (38.1)	75 (38.5)	NS
2	0 (0)	38 (21.0)	38 (19.5)	NS
3	2 (14.4)	68 (37.6)	70 (35.9)	NS
4	6 (42.8)	6 (3.3)	12 (6.1)	0.0003
Outcome of Grade 1 ulcers (1 month after enrollment)				
Complete healing	0 (0)	24 (13.3)	24 (32)	NS
Improved	4 (66.7)	29 (16.0)	33 (44)	NS
Worsening	2 (33.3)	16 (8.8)	18 (24)	NS

Values are median and interquartile ranges (25th to 75th percentile) or numbers and percentages. NS, non-significant.

In 12 strains, *edin-B* and *etD* genes were found to be associated. They were all positive for the *sarA* gene regulon, which can control *edin-B* expression [20]. Ten of these strains belonged to the *agrI* cluster. Five of these ten patients had a grade 4 DFU. Regarding the virulence profiles of these ten isolates, all the strains carried the following genes: the haemolysin-encoding genes (*hlgv*, *hla* and *hly*), the *egc* cluster of enterotoxins, the genes encoding leukocidin *lukDE*, the genes encoding intracellular adhesion proteins (*icaA*, *icaC* and *icaD*), *cap5*, and five genes encoding MSCRAMM (*clfA*, *clfB*, *fib*, *ebpS* and *fmbA*). Of note, *cna* and *fmbB* genes were absent whereas *tet* and *fosB* encoding resistance genes were always present.

As shown in Table 2, the *edin*-positive *S. aureus* isolates displayed a low clonal diversity compared with the other strains (40 CCs). None of the *edin*-positive isolates belonged to CC5/CC8. A total of three known CCs and one unknown singleton were identified. CC25/28 methicillin-sensitive *S. aureus* (MSSA) (*n* = 10, 76.9%) was the most common CC in *edin*-positive strains. This CC and the CC80-MRSA (European Community MRSA Clone, *n* = 3) were significantly associated with *edin*-positive isolates (*p* < 0.01). The *edin-B*-positive singleton isolated from a grade 4 ulcer was found to be negative for *etD*. It was identified as ST152-MSSA negative

for *lukDE* and *hlgv*. This reinforced the interest of using *edin-B*, together with the highly prevalent markers *lukDE* and *hlgv*, to decipher strains of *S. aureus* with a high potential for infection in DFI.

Discussion

DFUs are prone to infections that may spread systematically and lead to amputation of lower extremities and possibly to death. From a clinical point of view, differentiating colonization from infection may be problematic in DFUs due to the masking impact of the neuropathy and/or ischemia [15]. Monomicrobial DFI thus represents a unique model in humans to compare the distribution of bacterial factors between infecting and colonizing strains of *S. aureus*. Although the virulence potential of *S. aureus* relies on the combination of multiple factors, those associated with colonizing strains of grade 1 DFUs have probably a poor contributing role in virulence and notably in invasion. In this multicentre study, we found that among the 14 *edin*-positive *S. aureus* isolates, ten were recovered from deep grade 3 or 4 infections (at baseline or during follow-up). Moreover, in the four other *edin*-positive cases in which outcomes were favourable, the strains isolated at follow-up

TABLE 2. Main virulence profiles of *S. aureus* harbouring *edin* genes isolated from diabetic foot ulcers

	<i>edinA</i> n = 1	<i>edinB</i> n = 13	Other strains n = 181	p <i>edin</i> vs. other
Resistance markers				
<i>mecA</i>	0	2 (15.4)	37 (20.4)	NS
<i>tet</i>	1	13 (100)	177 (97.8)	NS
<i>fosB</i>	1	10 (76.9)	114 (63.0)	NS
Enterotoxins				
<i>sea</i>	1	1 (7.7)	78 (43.1)	0.04
<i>seb</i>	0	3 (23.1)	9 (5.0)	NS
<i>sei</i>	0	10 (76.9)	77 (42.5)	0.05
<i>egc</i> cluster	0	10 (76.9)	80 (44.2)	NS
Toxins				
<i>lukDE</i>	1	12 (92.3)	114 (63.0)	0.04
<i>hlgv</i>	1	12 (92.3)	95 (52.5)	0.003
<i>hlyB</i>	1	6 (46.2)	100 (55.2)	NS
<i>lukS-PV lukF-PV</i>	0	3 (23.1)	1 (0.6)	0.001
<i>etA</i>	0	0 (0)	0 (0)	NS
<i>etB</i>	0	0 (0)	0 (0)	NS
<i>etD</i>	0	12 (92.3)	0 (0)	<0.0001
Capsules				
<i>cap5</i>	1	12 (92.3)	98 (54.1)	0.004
<i>cap8</i>	0	1 (7.7)	83 (45.9)	0.004
MSCRAMM				
<i>fib</i>	1	12 (92.3)	87 (48.1)	0.001
<i>fmbA</i>	1	13 (100)	162 (89.5)	NS
<i>fmbB</i>	1	0 (0)	48 (26.5)	NS
<i>bbp</i>	1	11 (84.6)	147 (81.2)	NS
<i>ebpS</i>	1	13 (100)	181 (100)	NS
<i>clfA</i>	1	13 (100)	181 (100)	NS
<i>clfB</i>	1	13 (100)	181 (100)	NS
<i>sarA</i>	1	13 (100)	181 (100)	NS
Other virulence markers				
<i>isdA</i>	1	13 (100)	181 (100)	NS
<i>spIA</i>	1	12 (92.3)	112 (61.9)	0.02
<i>spIB</i>	1	12 (92.3)	109 (60.2)	0.01
<i>cna</i>	0	1 (7.7)	75 (41.4)	0.01
<i>chp</i>	0	10 (76.9)	102 (56.4)	NS
<i>agr</i>				
<i>agrI</i>	1	11 (84.6)	114 (63.0)	NS
<i>agrII</i>	0	0 (0)	42 (23.2)	0.04
<i>agrIII</i>	0	2 (15.4)	24 (13.3)	NS
<i>agrIV</i>	0	0 (0)	1 (0.6)	NS
Clonal complexes				
Unknown CC	1	0 (0)	18 (9.9)	NS
CC25/28-MSSA	0	10 (76.9)	0 (0)	<0.0001
ST152-MSSA	0	1 (7.7)	0 (0)	NS
CC80-MRSA	0	2 (15.4)	0 (0)	0.005
CC5-MSSA	0	0 (0)	14 (7.7)	NS
CC8-MSSA	0	0 (0)	36 (19.9)	NS
Others	0	0 (0)	113 (62.5) ^a	<0.001

^a20 other CCs determined in this group. NS, non-significant

were no longer positive for *edin* and were from different CCs. These findings suggest that these *edin*-positive strains were successfully removed during debridement, allowing colonization of wounds by other strains. At least, these findings clearly indicate that *edin* genes have a higher prevalence in *S. aureus* from high-grade ulcer isolates.

Three alleles of *edin* have been characterized [21–23]. They all share similar biochemical activities toward Rho proteins but are encoded within different genetic backgrounds. The gene encoding *edinA* is located on a plasmid, as for *edinC* and the closely associated exfoliative toxin-B encoding gene [22]. *edinB* is chromosomally encoded within a highly variable pathogenicity island in close proximity to exfoliative toxin-D encoding gene [20,23]. Although the DNA array we used did not efficiently detect the *edinC* gene (unpublished data), the absence of detection of the exfoliative type-D strongly suggests that *edinC*-positive strains were not present in this

type of infection. Thus, *edinB* is the most prevalent *edin* gene associated with DFUs.

The clonal complex analysis indicates that *edin*-positive strains belonged to four major groups: a singleton near CC8 (*edinA*, *n* = 1), a singleton belonging to ST152-MSSA (*edinB*, *n* = 1), CC80-MRSA (*edinB*, *n* = 2) and CC25/28-MSSA (*edinB*, *n* = 10). Here we identified an *edinB*-positive/*etD*-negative strain that belongs to ST152-MSSA. Importantly, this strain appeared negative for the haemolysin (*hlgv*) and the leukocidin (*lukDE*) genes, which have been defined as two highly prevalent virulence markers of infectious strains in DFU. Given that the *edinB*-positive ST152 strain of *S. aureus* was isolated from a Grade 4 ulcer, this result further emphasizes the interest in screening *edinB* as a surrogate maker to discriminate colonizing from infecting strains of *S. aureus* in DFU. It is worth noting that none of the *edin*-positive *S. aureus* belonged to CC5/CC8; two CCs claimed to be predictive of a favourable outcome [13].

Our analysis of the distribution of *edin* in DFU supports the idea that *edin* might work together with the arsenal of *S. aureus* virulence factors to give the bacteria a higher potential for infection [5, 12]. In keeping with this concept, previous studies indicated that EDIN factors might favour induction of deep-seated infectious foci [24,25]. *Edin* encoding genes thus represent additional markers of interest to differentiate infecting from colonizing *Staphylococcus aureus* strains in DFU and to predict the wound outcome. Our results highlight the potential usefulness of the molecular biology in patients in whom the clinical diagnosis of infection is made difficult by peripheral arterial disease, neuropathy or impaired leukocyte functions [26]. The DNA microarray might provide important help for clinicians and might allow adequate management of uninfected ulcers carrying *S. aureus*, according to its genotype profile. The detection of some CCs and virulent genes in grade I ulcers raises the question about antibiotic treatment in apparently uninfected DFU and challenges the dogma about abstention of antibiotic therapy in clinically uninfected ulcers. Indeed, the absence of CC5/CC8 corresponding to colonizing strains and the presence of CC25/28 and/or of *lukDE*, *hlgv* and *edin* genes suggest the need for antibiotic treatment as a therapeutic strategy. However, to date the techniques we used are largely in the field of research.

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Transparency Declaration

The authors have no conflicts of interest to declare.

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Travail n°4

First report of the predominance of clonal complex 398 *Staphylococcus aureus* strains in osteomyelitis complicating diabetic foot ulcers: a national French study

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Les ostéites à *Staphylococcus aureus* sont une complication fréquente (10 à 60%) des plaies du pied chez le diabétique. Or très peu de documentation sur ces souches n'était disponible. L'objectif de cette étude était donc d'évaluer les clones de *S. aureus* circulants en France dans cette pathologie.

Au cours du Programme Hospitalier de Recherche Clinique, une étude ancillaire a été élaboré dans le but de mieux connaître le potentiel de virulence et épidémiogène de ces souches de *S. aureus*. C'est ainsi que des patients adultes diabétiques de type 1 et 2, ayant une plaie infectée du pied (Grades 2-4 ; SPILF 2007) ont été inclus du 1/01/2008 au 31/12/2010 dans les 12 centres hospitaliers français. Après préparation de la plaie, un prélèvement était réalisé par biopsie osseuse selon les recommandations nationales. Les prélèvements monomicrobiens à *S. aureus* étaient conservés pour l'étude génotypique par des biopuces à ADN (StaphyType96®, Alere): détection de 330 gènes de virulence/résistance et assignation de l'origine clonale de la souche.

Au total, 57 patients ont été inclus (63% d'hommes, âge médian 67 ans (38-85)) et 61 souches de *S. aureus* ont été isolées (4 patients ayant 2 types de *S. aureus* de morphotypes différents). La prévalence des SARM était de 19,7%. Une grande diversité clonale a été observée : un total de 13 complexes clonaux (CC) connus et 4 singletons. Le clone ST398 (d'origine animale notamment porcine) était le plus fréquemment détecté (n=20, 33%) représentant 41% des souches sensibles à la méticilline. Ces souches ont été isolées dans 10 hôpitaux sur 12. Aucun lien n'a pu être démontré entre les différents patients suggérant une propagation nationale de ce clone. Ces souches ont la particularité d'être productrices de pénicillinase et sensibles aux autres antibiotiques. Quelques caractéristiques génétiques ont été observées : absence d'entérotoxine, présence d'hémolysines (*hlgv*, *hlgA*, *hla*, *hld*), de *cap5*, et de 3 gènes codant pour une MSCRAMM (*bbp*, *clfA*, *clfB*). Toutes les souches appartenaient au groupe *agr* 1.

Cette étude multicentrique a démontré l'émergence et la dissémination du clone ST398 dans les ostéites du pied chez le diabétique en France. L'adaptation d'un clone passé de l'homme à l'animal est un problème alarmant nécessitant une surveillance accrue.

First report of the predominance of clonal complex 398 *Staphylococcus aureus* strains in osteomyelitis complicating diabetic foot ulcers: a national French study

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Abstract

Staphylococcus aureus is the most common pathogen cultured from diabetic foot infection including diabetic foot osteomyelitis. This French multicentre study determined the genetic content of *S. aureus* isolated from 157 consecutive cases admitted to 12 diabetic foot centres between 2008 and 2011. We describe for the first time the emergence of the CC398 methicillin-susceptible *S. aureus* clone, the main clone in diabetic foot osteomyelitis, and its tropism for bone. This clone spreads to humans from an animal source through its intrinsic virulence. This adaptation of *S. aureus* isolates looks to be a worrisome problem and should be carefully monitored.

Keywords: Clonal complex 398, diabetic foot, methicillin-susceptible *Staphylococcus aureus*, osteomyelitis

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Foot ulcers are common in diabetic patients, with a prevalence as high as 25% [1]. These ulcers frequently become infected, and spread of infection to soft tissue and bony structures is a major causal factor for lower-limb amputation [2], making early diagnosis and adequate treatment essential. Depending on the setting and severity of infection, prevalence of bone involvement widely ranges from 10 to 60% [3]. Chronic ulcers of the foot are at high risk for infection and secondary spread to underlying bone structures [4]. Consequently, all factors that contribute to delayed wound healing may increase the risk for developing diabetic foot osteomyelitis (DFOM). Although *Staphylococcus aureus* is the most common pathogen cultured from bone in DFOM in western countries [5,6], there is currently no report on the clones of this microorganism in this condition. *Staphylococcus aureus* clonal complex (CC) 398, which is commonly found as a colonizing strain in pig farming, is emerging as a cause of human infections. In recent years, increasing numbers of studies throughout the world have identified CC398 strain as a livestock colonizing strain, but continuously increasing and alarming cases of human infections have been reported [7]. The present study reports for the first time the emergence of CC398 strains in a French cohort of patients with diabetic foot infections (DFI) and its tropism for bone.

Between 1 January 2008 and 31 December 2010, we prospectively and consecutively screened outpatients attending one of the 12 participating French foot clinics for DFI. The local ethics committee (South Mediterranean III) approved this study. After informed consent was obtained, only patients with a documented DFI were included if they had not received any antibiotic agents in the previous month. Patients were suspected of having osteomyelitis of the foot if they had at least two of the following clinical criteria: (1) a wound with duration ≥ 2 weeks, located over an underlying bony prominence, with an area >2 cm² or a depth >3 mm; (2) a positive probe-to-bone test; and (3) abnormalities consistent with the diagnosis of osteomyelitis either on plain X-rays, radionuclide procedures (three-phase bone scan and/or labelled leucocyte imaging) or magnetic resonance imaging. After wound debridement, samples for bacterial culture were obtained by swabbing the wound base, needle aspiration or tissue biopsy for skin and soft tissue infection (SSTI) and by transcutaneous bone biopsy using a previously described procedure [5]. Only patients with monomicrobial culture for *S. aureus* were included. Genus, species and antibiotic susceptibilities were determined using the Vitek 2 card (BioMérieux, Marcy-l'Etoile, France) and interpreted according to the recommendations of the French Society for Microbiology (<http://www.sfm-microbiologie.org>). The Alere StaphyType DNA microarray was used according to protocols that have been previously detailed [8]. This technology allowed the identification of the clonal complex

(CC) of strains. The affiliation of isolates was assessed by an automated comparison of hybridization profiles to a collection of previously characterized reference strains [8]. Screening for the presence of prophage ϕ Sa3 (*scn*, *chp*, *pvl*, *tetM*) in CC398 was obtained by DNA array [9,10]. The presence of each clonal complex in *S. aureus* strains was compared according to the clinical condition (DFOM or SSTI) using Fisher's exact test. Statistical analysis was performed using the S-Plus 2000 software package (Insightful Corporation, Seattle, WA, USA) and results were considered significant for $p < 0.05$.

During the study period, 157 patients (Table 1) were recruited, in whom *S. aureus* was the sole organism isolated from the bacterial culture of their DFI. Seventy-seven wounds (49%) were associated with osteomyelitis and 80 with SSTI without involving the bone. Among the 77 patients, 81 *S. aureus* isolates were detected; two different *S. aureus* strains (α -haemolytic and β -haemolytic colonies) were isolated from four patients. Prevalence of methicillin-resistant *S. aureus* (MRSA) was identical in cultures from DFOM ($n = 13$, 16%) and from SSTI ($n = 8$, 10%). As shown in Table 2, the *S. aureus* isolates displayed a high clonal diversity. A total of 18 known CCs and 11 singletons were identified. CC398 methicillin-sensitive *S. aureus* (MSSA; $n = 31$) was the most frequently found CC in DFOM with a prevalence of 38.3% and was

significantly associated with this condition ($p < 0.001$). Of the MSSA strains, 45.6% belonged to the CC398 clone. On the other hand, CC45 MSSA ($n = 13$, 16.3%) was significantly associated with SSTI ($p < 0.05$). The antibiotic susceptibilities showed that the CC398 strains were susceptible to all tested antibiotics except penicillin G. The strains were isolated in 10 of the 12 foot clinics. No link could be found between each patient harbouring this clone, suggesting a national propagation of this strain in DFOM. No relation with animal farming density or seasonal effect was noted; the clone was distributed throughout the study period. Interestingly strains with different appearances on blood agar media had the same gene content. Regarding the MRSA strains isolated from DFOM, they mainly belonged to three CCs: CC8 MRSA (Lyon Clone, $n = 9$), the main clone present in French hospitals, CC5 MRSA (New Paediatric, $n = 2$) and ST22 MRSA (Barnim Epidemic Strain, UK-EMRSA-15, $n = 1$). CC398 strains were characterized by some distinctive features regarding the distribution of virulence factors: absence of enterotoxins, presence of haemolysins, and genes encoding intracellular adhesion proteins, *cap5*, together with three genes encoding MSCRAMM (*bbp*, *clfA*, *clfB*). All the isolates were found in *agr* group I. Moreover, the CC398 strains belonged to human MSSA clades carrying the prophage ϕ Sa3 due to the presence of genes encoding human-specific immune evasion cluster (*chp*, $n = 29$ /

TABLE 1. Demographic and clinical characteristics of study patients

Characteristics	Patients with CC398 strains <i>n</i> = 31	DFOM <i>n</i> = 77	SSTI <i>n</i> = 80	Total <i>n</i> = 157
Age (range), years	66 (38–91)	66 (38–85)	66.5 (33–101)	66.5 (33–101)
Male/female, <i>n</i> (%)	22 (71)/9 (29)	58 (75.3)/19 (24.7)	58 (72.5)/22 (27.5)	116 (73.9)/32 (26.1)
Type 1/type 2 diabetes mellitus	4 (12.9)/27 (87.1)	7 (9.1)/70 (90.9)	14 (17.5)/66 (82.5)	21 (13.4)/136 (86.6)
HbA1c (%)	8.5	8.4	8.4	8.4
Cardiovascular disease				
Absence	9 (29.0)	23 (29.9)	16 (20)	39 (24.8)
Coronary heart disease	9 (29.0)	23 (29.9)	31 (38.8)	54 (34.4)
Peripheral arterial disease	18 (58.1)	45 (58.4)	57 (71.3)	102 (65.0)
Arterial hypotension	25 (80.6)	60 (77.9)	65 (81.3)	125 (79.6)
Stroke	1 (3.2)	5 (6.5)	5 (6.3)	10 (6.4)
Nephropathy				
Absence	6 (19.4)	15 (19.5)	29 (36.3)	44 (28.0)
Microalbuminuria	6 (19.4)	11 (14.3)	26 (32.5)	37 (23.6)
Proteinuria	10 (32.3)	28 (36.4)	20 (25)	48 (30.6)
Renal failure	9 (29.0)	23 (29.9)	28 (35)	51 (32.5)
Neuropathy				
Peripheral	31 (100)	77 (100)	75 (93.8)	152 (96.8)
Autonomic	7 (22.6)	12 (15.6)	20 (25)	32 (20.4)
Diabetic retinopathy				
Absence	12 (38.7)	32 (41.6)	31 (38.8)	63 (40.1)
Non-proliferative diabetic retinopathy	13 (41.9)	33 (41.6)	40 (50)	73 (46.5)
Proliferative diabetic retinopathy	6 (19.4)	12 (15.6)	10 (12.5)	22 (14.0)
Lifestyle factors				
Obesity	12 (38.7)	31 (40.3)	32 (40)	63 (40.1)
Smoking	8 (25.8)	19 (24.7)	22 (27.5)	41 (26.1)
Alcoholism	5 (16.1)	12 (15.6)	14 (17.5)	26 (16.5)
Sedentary	25 (80.6)	60 (77.9)	62 (77.5)	122 (77.7)
First wound/Recurrence	8 (25.8)/23 (74.2)	17 (22.1)/60 (77.9)	19 (23.8)/61 (76.2)	36 (22.9)/121 (77.0)
IWGDF-IDSA grade				
2 (Mild)	0 (0)	0 (0)	38 (47.5)	38 (24.2)
3 (Moderate)	24 (77.4)	54 (70.1)	42 (52.5)	96 (61.1)
4 (Severe)	7 (22.6)	23 (29.9)	0 (0)	23 (14.7)

DFOM, diabetic foot osteomyelitis; HbA1c, glycated haemoglobin; IDSA, Infectious Diseases Society of America; IWGDF, International Working Group of the Diabetic Foot; SSTI, skin and soft tissue infection.

Values are median and interquartile ranges (25th–75th percentile) or numbers and percentages.

TABLE 2. Distribution of clonal complexes among *Staphylococcus aureus* strains isolated from diabetic foot osteomyelitis (DFOM) and skin and soft tissue infection (SSTI) in diabetic foot ulcers in a French population

Clonal complexes	DFOM n = 81	SSTI n = 80	Total n = 161	p DFOM vs SSTI
CC398-MSSA	31 (38.3)	4 (5)	35 (21.7)	<0.001
CC7-MSSA	4 (4.9)	14 (17.5)	18 (11.2)	NS
CC8-MRSA	9 (11.1)	6 (7.5)	15 (9.3)	NS
CC45-MSSA	2 (2.5)	13 (16.3)	15 (9.3)	0.05
CC30-MSSA	5 (6.2)	9 (11.3)	14 (8.7)	NS
None ^a	7 (8.6)	3 (3.8)	10 (6.2)	NS
CC18-MSSA	0 (0)	9 (11.3)	9 (5.6)	NS
CC59-MSSA	6 (7.4)	1 (1.3)	7 (4.3)	NS
CC25/28-MSSA	0 (0)	7 (8.8)	7 (4.3)	NS
CC5-MSSA	4 (4.9)	3 (3.8)	7 (4.3)	NS
CC8-MSSA	3 (3.7)	0 (0)	3 (1.9)	NS
CC5-MRSA	3 (3.7)	0 (0)	3 (1.9)	NS
CC9-MSSA	0 (0)	3 (3.8)	3 (1.9)	NS
CC1-MSSA	0 (0)	3 (3.8)	3 (1.9)	NS
CC15-MSSA	3 (3.7)	0 (0)	3 (1.9)	NS
CC101-MSSA	0 (0)	2 (2.5)	2 (1.2)	NS
CC7-MRSA	0 (0)	2 (2.5)	2 (1.2)	NS
CC97-MSSA	2 (2.5)	0 (0)	2 (1.2)	NS
CC22-MSSA	1 (1.2)	1 (1.3)	2 (1.2)	NS
CC22-MRSA	1 (1.2)	0 (0)	1 (0.6)	NS

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*.

^aNone, the clonal complexes of these strains are not known and are absent in databanks. They correspond to different singletons. NS, not significant.

31 and *scn*, *n* = 30/31) and absence of *pvl* and *tetM* (associated with livestock isolates).

The present French multicentre study highlighted for the first time the emergence and spread of CC398 clone in DFI and its tropism for bone. For many years, this clone has been described in various livestock and most studies reported infection with MRSA. As an example, swine have been identified as a major reservoir for CC398 MRSA isolates [11]. Various clinical infections with livestock-associated CC398 MRSA have been reported around the world [11–13]. Recently, the emergence of an animal-independent CC398 MSSA clone has been documented in several countries. However, Price *et al.* [9] reported that the human CC398s were the original CC398 clone from which the livestock-associated clones emerged. Our strains had a human origin, indicated by the presence of the human innate immunomodulatory genes carried by ϕ Sa3 prophage that plays crucial roles in human niche adaptation [9,10]. The limited surveillance of MSSA has precluded an accurate assessment of the global spread of CC398 [10]. Hence, this emergence was described in community households in northern Manhattan [12]. Since that case, some reports have described CC398 MSSA not as a colonizer but as an infectious agent including cases of necrotizing pneumonia and invasive bloodstream infections in young healthy individuals [14,15]. The concern that these strains may represent a more virulent CC398 subtype is further supported by the higher prevalence of these strains in bloodstream infections of patients who had no documented

exposure to livestock [14]. In France this emergence was also described in bloodstream infections [16]. In addition to the intrinsic virulence exhibited by CC398 MSSA in previous studies, the potential to acquire other virulence factors such as Pantón–Valentine leukocidin [17,18], as well as resistance to multiple classes of antimicrobial drugs [9] warrants reinforced surveillance. Another animal clone, *S. aureus* CC97, was detected in our isolates. This clone was shown to have a better adaptation to animals than to humans and has been associated with bovine mastitis [19] with little impact on humans [20]. In the present study, the two CC97 *S. aureus* strains were isolated in DFOM as previously observed [21], suggesting a bone tropism of these clones of animal origin. The adaptation of animal *S. aureus* isolates to humans is a worrisome problem and should be carefully monitored.

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Transparency Declaration

The authors declare no conflicts of interest.

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Travail n°5

Existence of a colonizing *Staphylococcus aureus* strain isolated in diabetic foot ulcers

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S. aureus, bactérie opportuniste responsable d'un large éventail de pathologies, est également un commensal des fosses nasales et d'autres muqueuses. Dans les plaies chroniques, nous avons montré la coexistence de deux populations de *S. aureus*: une colonisante et une infectante. Le but de cette étude était de caractériser les souches colonisantes de *S. aureus*.

Dans le but de déterminer l'organisation génomique des souches colonisantes/infectantes isolées de plaies de pied chez des diabétiques, nous avons utilisé la technique des Optical maps (OpGen) en utilisant les cartes de restriction *Xba*I. L'insertion génétique observée a été caractérisée par pyroséquençage. L'impact de cette insertion sur le métabolisme du Fer a été évalué par qRT-PCR après extraction d'ARNm à partir de cultures bactériennes en présence/absence de Fer et test au chromazurol. La virulence des souches a été étudiée sur les modèles *C. elegans* et zebrafish. La prévalence de cette insertion a été évaluée sur un souchier national de *S. aureus* isolés de pieds diabétiques.

En étudiant les profils des Optical maps sur notre collection de souches de virulence définie, nous avons localisé et caractérisé une insertion génétique d'ADN présentes uniquement chez les souches colonisantes. Le séquençage de la zone a permis d'objectiver l'insertion d'un phage au niveau de la région hotspot Φ NM2 près du locus *isd*, le principal déterminant du transport membranaire du Fer. Cette insertion est responsable d'une dérégulation de l'activité de Fur, régulateur de l'acquisition du Fer et du biofilm chez *S. aureus*. Elle réduit significativement la virulence de la bactérie dans les 2 modèles in vivo. L'expulsion du phage permet de restaurer la régulation du locus *isd*, la production de sidérophore, la formation de biofilm et la virulence bactérienne. La prévalence de ces souches colonisantes étaient de 20% (39/195) dont 89% appartenaient au complexe clonal CC8.

Les souches colonisantes du fait de leur faible potentiel de virulence pourrait être détectées afin de contribuer à une meilleure utilisation des antibiotiques, un point essentiel face à l'augmentation constante de la multirésistance.

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Existence of a Colonizing *Staphylococcus aureus* Strain Isolated in Diabetic Foot Ulcers

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Staphylococcus aureus is an opportunistic bacterium capable of causing a wide range of severe diseases when it gains access to underlying tissues. Paradoxically, *S. aureus* is a common inhabitant of the skin microflora and colonizes the nares and other human mucosa. The purpose of this study was to determine the genetic basis for the differences in the pathogenic versus colonizing potential of *S. aureus* isolated from diabetic foot ulcers (DFUs). By performing optical map comparisons of a collection of *S. aureus* strains isolated from DFUs, we brought to light a prophage present in noninfecting bacteria. The phage, namely ROSA-like, was localized in a hotspot region Φ NM2 near the locus *isd*, the iron surface determinant system. The integrated phage significantly reduces the virulence of the strain and increases the biofilm formation. DFUs seem to be a specific niche of this colonizing strain. The ROSA-like phage represents the first description of a mobile element present mainly in *S. aureus* isolated from DFUs, which modulates the relationship of the bacteria with its human host. This phage appears to attenuate bacterial virulence and promote colonization.

Staphylococcus aureus is by far the most common and virulent pathogen in diabetic foot infection (1,2). However, this causative pathogen is a common inhabitant of the skin microflora and colonizes the nares and other human

mucosa. It may be considered as an opportunistic colonizing organism. Recently, we demonstrated the coexistence of two populations of *S. aureus* strains isolated from diabetic foot ulcer (DFU): strains isolated from uninfected ulcers with a low virulence potential as opposed to strains isolated from infected ulcers with a high virulence potential (3,4). Moreover the strains belonged to two clonal complexes (CC8/CC5) that appeared to be linked to uninfected ulcers, enabling us to distinguish uninfected from infected wounds (5). In this study, we describe for the first time an insertion of a phage in the CC8 lineage of methicillin-sensitive *S. aureus* (MSSA), which is associated with the colonizing *S. aureus* strains, and we report the impact of this phage on biofilm formation and bacterial virulence.

RESEARCH DESIGN AND METHODS

Bacterial Strains and Plasmids

All bacterial strains used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria Bertani broth or brain-heart infusion broth.

Caenorhabditis elegans and Zebrafish Killing Models

Fer-15 worms were maintained and infected as previously described (6). All experiments were conducted in triplicate and repeated at least five times for each strain. *S. aureus* virulence was assessed using the nematode survival curve

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See accompanying article, p. 2701.

Table 1—LT50 of *C. elegans* infected by the different *S. aureus* strains and evaluation of CFU of each strain in the *C. elegans* digestive tract

Strain	Characteristics of the <i>S. aureus</i> strain	LT50 in days (95% CI inf-sup)	<i>P</i> NSA1385 vs. other strains	Median CFU [range] in nematode after 72 h
NSA1385†	Clinical, strain isolated from colonized DFU (grade 1)	4.3 (4.0–4.6)	—	2.2×10^5 [$1.0\text{--}3.4 \times 10^5$]
NSA1322†	Clinical, strain isolated from colonized DFU (grade 1)	4.0 (3.7–4.4)	NS	6.1×10^5 [$4.9\text{--}6.6 \times 10^5$]
NSA739†	Clinical, strain isolated from infected DFU (grade 3)	1.7 (1.4–2.0)	<0.001	5.0×10^5 [$4.8\text{--}5.2 \times 10^5$]
NSA18026†	Clinical, strain isolated from infected DFU (grade 4)	1.8 (1.5–2.2)	<0.001	5.5×10^5 [$4.8\text{--}6.1 \times 10^5$]
NSA1385(P–)	Strain isolated from colonized DFU (grade 1) after excision of the phage	1.6 (1.3–1.8)	<0.001	4.2×10^5 [$3.8\text{--}4.4 \times 10^5$]
Newman	Reference	4.6 (4.4–4.9)	NS	4.9×10^5 [$4.2\text{--}5.6 \times 10^5$]
OP50	Control strain	7.8 (7.5–8.1)	<0.001	—

The results are representative of at least five independent trials for each group of strains. *P* is a pairwise comparison using a log-rank test. †Sotto et al. (4). inf-sup, inferior-superior.

and calculating the LT50 and LT100 (median lethal time 50% and 100%, respectively).

The presence of *S. aureus* in the *Caenorhabditis elegans* digestive tract was determined at 72 h as described by Garsin et al. (7). Three replicates were performed for each strain. Infection of zebrafish embryos was carried out as previously described (8). More information regarding the two models can be found in the Supplementary Data.

Optical Maps

Twenty-two strains from our panel of colonizing and infecting *S. aureus* strains isolated from DFUs (5) were chosen for optical mapping. Optical maps were provided by OpGen (Gaithersburg, MD), prepared on the Argus Optical Mapping System as described previously (9), and analyzed with the support of Phylogene (Bernis, France). The optical maps of the studied strains were then compared with the in silico restriction maps of 19 sequenced *S. aureus* isolates whose sequence genomes were available in GenBank and transformed by using the MapSolver v.2.1.1 software (OpGen SA).

Sequencing of the NSA1385 Strain and the 44-kb Insertion

Genomic DNA of *S. aureus* NSA1385 was sequenced using a 454 Life Sciences-Roche platform by Lifesequencing S.L. (Valencia, Spain). The combination of scaffolds and contigs resulted in an estimated genome size of 3.2 Mb.

PCR for the Detection of Phage Insertion/Deletion and Sequencing

The PCR protocol is presented in the Supplementary Data. After purification, PCR products were sequenced using a PerkinElmer ABI 377 sequencer and compared with sequences in GenBank by BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

Biofilm Formation

To evaluate the biofilm formation, we used the BioFilm Ring Test (BioFilm Control, Saint Beauzire, France) according to

the manufacturer's recommendations (10). Three experiments with three repeats each (three wells per slide) were performed per strain and incubation time.

Evaluation of Spontaneous Phage Excision

To detect and evaluate spontaneous excision of the ROSA-like phage from the hotspot region Φ NM2, we used different procedures already described: the TMS medium with or without FeCl₃ (50 μ mol/L) to create iron-repleted and iron-restricted growth conditions, mitomycin C, and UV treatment (11–13). We evaluated the occurrence frequency of NSA1385 mutants that lost the phage by counting the number of NSA1385 without phage colony-forming unit (CFU) and compared with the NSA1385 CFU number. Phage excision was confirmed using a PCR assay and genome sequencing.

Statistical Analysis

The Mann-Whitney test was used to compare the in vivo bacterial growth of the different strains. To compare overall survival curves in nematode and zebrafish killing assays, a Cox regression was used. For pairwise comparison of two survival curves in nematode and zebrafish killing assays, we used a log-rank test. Statistical analysis was performed using the S-PLUS 2000 software package (Insightful Corporation, Seattle, WA), and results were considered significant at $P < 0.05$.

RESULTS

Noninfecting Strains Are Less Virulent Than Infecting Strains

We used two infection models to confirm our previous observations that clinical *S. aureus* strains isolated from grade 1 DFUs are less virulent than strains isolated from grade 2–4 DFUs (4). We analyzed the behavior of five clinical strains: the two noninfecting strains (NSA1322 and NSA1385 from grade 1 DFU), the two infecting strains (NSA739 and NSA18026 from grade 2–4 DFU),

and the reference strain Newman. In the *C. elegans* model, the five studied strains killed the nematodes more rapidly than the avirulent *Escherichia coli* OP50 strain used as nutrient for the nematodes ($P < 0.001$) (Table 1). The LT50 were similar for the two colonizing strains and the strain Newman but significantly longer ($P < 0.001$) than the LT50 of the infecting strains ($4.0\text{--}4.6 \pm 0.3$ vs. $1.6\text{--}1.7 \pm 0.2$ days, respectively) (Table 1). The differences in virulence were not due to differences in the survival and proliferation of strains within the nematode intestine, since the intestine colonization by the different strains was not significantly different (Table 1).

Based on the results obtained with *C. elegans*, we investigated whether the two uninfected strains (NSA1322 and NSA1385) also exhibit lower virulence in zebrafish embryos. We compared mortality rates over a 92-h period. The two colonizing strains caused less embryo deaths than the two infecting strains ($P < 0.001$) (Fig. 1A and B).

Nonvirulent Colonizing *S. aureus* Strains Carry a Genetic Island

To investigate the difference between colonizing and infecting strains isolated from DFUs (4), we used optical mapping to analyze their genome organization (Supplementary Fig. 1). The five colonizing strains were clonal ($>99\%$ similarity) and clustered closely to two reference strains (Newman and NCTC8325) with $\sim 98\%$ similarity. The majority of the infecting strains belonged to different clonal groups with $<90\%$ similarity to the colonizing strains. Interestingly, five infecting strains (NSA739, NSA6759, NSA11260, NSA18026, and NSA56348) exhibited 96.5% similarity with the colonizing strains. All these strains belonged to the CC8-MSSA clonal complex.

The major difference between the colonizing and the infecting CC8-MSSA strains was the presence of a large insertion located exclusively in all of the colonizing strains (CC8- and CC5-MSSA). The insertion was located at the previously described Φ NM2 integration hotspot (Supplementary Fig. 2), a known hotspot for genetic insertions, with phage insertions identified in the published

genomes of the two reference strains (Newman and NCTC8325) (14).

The 44-kb Genetic Island Corresponds to a ROSA-Like Phage

To characterize the genetic insertion present in the colonizing strains, a draft genome sequence of NSA1385 was determined to $\sim 29\times$ coverage. The integration site is in the intergenic region between *rpmF* (encoding the 50S ribosomal protein L32) and *isdB* (encoding the staphylococcal hemoglobin receptor required for heme-iron uptake). The insertion has a high G+C content (35.4%, compared with 32% for the chromosome in total) and appears to be an integrated bacteriophage. This phage has a genome of 44,031 bp with 73 open reading frames and is allocated within the unclassified dsDNA phages group. The comparison of the phage sequence with sequences in GenBank highlighted that the phage integrates at the same sequence as the phage ROSA, a previously described phage (15) with no known function. The sequences were comparable but we noted an inversion of a part of the phage sequence (Supplementary Fig. 2). We named this genetic island ROSA-like. More information can be found in the Supplementary Data.

Genetic Island Is Associated With Colonization of Chronic Wounds in France

To evaluate whether the ROSA-like phage insertion is a common feature from uninfected strains, we tested a collection of *S. aureus* strains isolated in different clinical situations in France (Table 2). The insertion was identified in 40 of 392 strains (10.2%) of our collection, with 39 of 75 (52%) from colonizing DFUs and 1 of 131 (0.8%) from nose. The ROSA-like phage was almost exclusively found in grade 1 ulcers (39 of 44 uninfected wounds, 88.6%), and all the strains harboring the insertion belonged to the CC8/CC5-MSSA lineages.

Genetic Island Promotes Biofilm Formation

To determine the impact of the genetic insertion, we studied biofilm formation using the BioFilm Ring Test. In

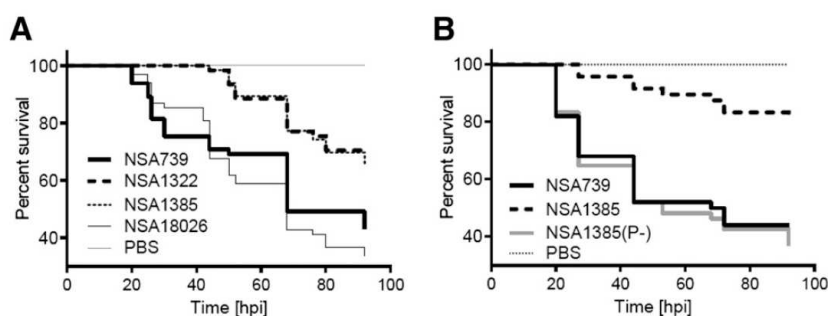


Figure 1—Virulence of the different *S. aureus* strains in zebrafish embryos. A: Survival curves of zebrafish embryos after infection with two infecting (NSA739 and NSA18026) and two colonizing (NSA1385 and NSA1322) strains. B: Survival curves of zebrafish embryos after infection with NSA739, NSA1385, and NSA1385(P-) strains. hpi, hours post-infection.

Table 2—Distribution of ROSA-like phage in a collection of *S. aureus* isolated in France

	Isolation site	No. of strains	No. of CC8/CC5 strains	No. of strains with phage ROSA-like insertion	No. of CC8/CC5 strains with phage ROSA-like insertion
Infection	DFI	120	6	0 (0%)	0 (0%)
	Acute cutaneous infection	10	2	0 (0%)	0 (0%)
	SSTI†	9	2	0 (0%)	0 (0%)
	Bacteremia	8	3	0 (0%)	0 (0%)
	Endocarditis	8	2	0 (0%)	0 (0%)
	Pneumonia	20	3	0 (0%)	0 (0%)
	Cystic fibrosis sputum	11	3	0 (0%)	0 (0%)
Colonization	Nose carriage	131	16	1 (0.8%)	1 (6.3%)
	DFU	75	39	39 (52%)	39 (100%)
Total		392	76	40 (10.2%)	40 (52.6%)

DFI, diabetic foot infection. †SSTI (skin and soft tissues infections): cellulitis ($n = 4$), necrotizing fasciitis ($n = 3$), and abscess ($n = 2$).

brain-heart infusion medium, we observed that the colonizing NSA1385 strain formed biofilms more rapidly than the infecting NSA739 strain (150 vs. 180 min) (Fig. 2). These results confirmed the colonizing role of the investigated strains.

ROSA-Like Phage Is Very Stable in Lysogens

An important issue in clinical practice is to know if the ROSA-like phage is stable or not. Using different DNA-damaging stimuli, we observed that the frequency of excision was $<5 \times 10^{-9}$, suggesting a strong selective pressure for its maintenance. One strain with a confirmed loss of the ROSA-like phage (detected by PCR, optical map, and DNA sequencing) was selected for further experiments and named NSA1385(P-).

Excision of the ROSA-Like Phage Restores Biofilm Formation and Bacterial Virulence

To definitively understand the impact of the phage insertion in the colonizing strain, we evaluated the biofilm formation and the virulence of NSA1385(P-). We observed the restoration of biofilm formation (Fig. 2) and bacterial virulence using the two in vivo models (Table 1 and Fig. 1B). All these data suggest that the ROSA-like phage clearly influences the virulence of the colonizing *S. aureus* strain. The absence of the phage restores the bacterial virulence. However, the low level of excision indicates that the ROSA-like phage is very stable and suggests that this colonizing strain does not require antibiotic treatment.

DISCUSSION

S. aureus is one of the most frequent pathogens isolated from community-acquired and nosocomial infections and is the most prevalent in DFUs (2). Even though *S. aureus* strains can colonize different human mucosa and may be considered as a commensal organism, these bacteria are clearly pathogens. However, we recently described the existence of a colonizing *S. aureus* strain isolated from DFUs (4). Here, a comparative genomic strategy using a collection of clinical strains detected a genetic determinant (a ROSA-

like phage) associated with the attenuation of the clonal group. To confirm that this element was responsible for the colonizing behavior, we assessed the distribution of the ROSA-like phage in disease-causing and asymptotically carried *S. aureus* in a national, nonbiased population taken from national epidemiological studies. The results demonstrated, for the first time, that the prophage was associated with the ability of the bacteria to colonize chronic ulcers and was responsible for the non-invasive character.

The carriage of virulence determinants by phage is not an uncommon situation in bacterial pathogens (16–20). In *S. aureus*, many of the phages encode and disseminate potent staphylococcal virulence factors (e.g., Panton-Valentine leukocidin) or resistance determinants (SCCmec cassette) (21). However, more recently, authors have shown that some phages could affect bacterial virulence by preventing the production of toxins (22). In our study, we demonstrated that the phage insertion blocks the virulence potential. Moreover, in the colonizing *S. aureus* strain, the difficulties to induce phage excision (using DNA-damaging agents), the low phage excision frequency, and the different

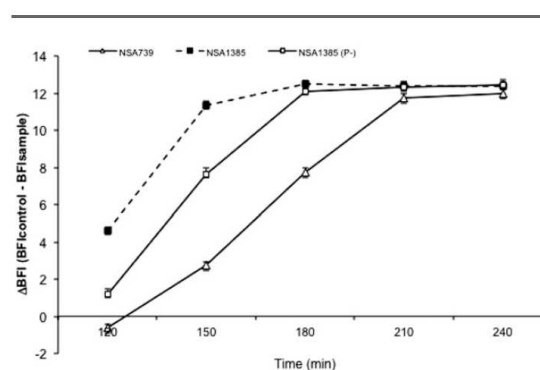


Figure 2—Kinetics of biofilm formation for the different *S. aureus* strains. Lines represent the SDs from three independent experiments with triplicate for each one. BFI, BioFilm Formation Index.

rearrangements noted (compared with the published genome sequence of phage ROSA) demonstrated a great stability of this insertion, suggesting a low ability to spread or transfer to other staphylococci.

The association with a high potential of biofilm formation and the avirulence of the *S. aureus* strains containing the ROSA-like phage may explain the ability of the bacteria to colonize chronic wound tissues and their ability to exist in the commensal state. Interestingly, these colonizing strains were exclusively found in uninfected ulcers that represent the niche of these strains. The role played by these bacteria would be directed toward the establishment of colonizing wounds and/or help other pathogens in the pathogenic process, the ROSA-like insertion bringing a selective advantage to this role. From a clinical point of view, the stability of phage and the very low potential of virulence of the colonizing strain suggest that it is not necessary to treat ulcers carrying this *S. aureus* type. Our findings may contribute to better diagnosis and improved treatment of diabetic ulcers.

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SUITE Travail n°5

The aim of this part was to document the impact of the phage ROSA-like in the iron metabolism of the colonizing strain.

Materials and methods

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria Bertani broth (LB) or Brain Heart Infusion broth (BHI). To obtain iron depleted medium (LB-Chelex and BHI-Chelex), LB or BHI was incubated with Chelex-100 (Sigma) resin (6% w/v) for 4h on a magnetic stirrer, and then filter (0.2 mm) sterilized. This procedure was repeated five times to reduce the iron concentration. Erythromycin or tetracycline were added to the growth medium when necessary to maintain recombinant plasmids.

A targeted knockout of *isdD* was created in *S. aureus* Newman strain as previously described. Complementation of the mutant was made by cloning the *isd* gene in a plasmid pSK265::*PlukS*(EcoRV) under the control of leukocidin promotor P-*lukS*. The IsdD coding region was amplified using primers *isd4836* and *isd5929* (Tables 1 and 2).

Introduction of the *fur* mutation into the colonizing NSA1385 strain was achieved by phage transduction, performed as previously described (Novick RP, 1991), from *S. aureus* 8325-4 *fur* mutant (Horsburgh MJ et al, 2001) using Φ11 as the transducing phage.

Table 1. Primers used in the study.

Name	Primers	Size
Rpm-F	5'-TGGCAGTACCAAAAAGAAGAACTTC-3'	490 bp
Isd-R	5'-GACACACTCAAAGCCAAAACAATAA-3'	
Isd4836*	5'-GTGATATCATGAGAAATGTAAACAAATTGC-3'	1093 bp
Isd5929*	5'-TTAGGATCCTCAAACCTTGGTTACC-3'	
GyrB-F	5'-AGTAACGGATAACGGACGTGGTA-3'	150 bp
GyrB-R	5'-CCAACACCATGTAAACCACCAGAT-3'	
IsdA-F	5'-AAGATAATGAACCTAAAAGGGC-3'	110 bp
IsdA-R	5'-GCCTAAACAAGCATCTAAAGTAAA-3'	

IsdB-F	5'-TGTCATCTTTTTGGCTGTCTTG-3'	139 bp
IsdB-R	5'-GCCAATACCGATAAATCTAACA-3'	
IsdD-F	5'-GCATGTTAAAACGTTGGCTG-3'	108 bp
IsdD-R	5'-GGCACTTCTTTTGCATCATC-3'	
Fur-F	5'-GGTTGGCGACAGTATACAGC-3'	288 bp
Fur-R	5'-ACATTCCATACATACTAAATGATG-3'	
XerD-F	5'-AAGGACTAAGTTCTAACACAATTGG-3'	300 bp
XerD-R	5'-CTTCGTCAACATTTAAAACG-3'	
Sbi-F	5'-AATATATCTCGAAGTTGCTAGTTGG-3'	469 bp
Sbi-R	5'-GCTTTATCCGCATTTTCAAT-3'	

*Enzymatic sites underlined and modified nucleotides in bold

Table 2. Main characteristics of the *S. aureus* strains used in the study

Strains names		Characteristics	References
Newman	Reference strain		-
NSA739	<i>S. aureus</i> strain isolated from infected DFU (Grade 3)		(Sotto et al., 2008)
NSA18026	<i>S. aureus</i> strain isolated from infected DFU (Grade 4)		(Sotto et al., 2008)
NSA1322	<i>S. aureus</i> strain isolated from colonized DFU (Grade 1)		(Sotto et al., 2008)
NSA1385	<i>S. aureus</i> strain isolated from colonized DFU (Grade 1)		(Sotto et al., 2008)
NSA1385(P-)	<i>S. aureus</i> strain isolated from colonized DFU (Grade 1) after excision of the phage		This study
1385Dfur	1385Dfur::tet, NSA1385 strain with a deletion of <i>fur</i> gene		This study
1385(P-)Dfur	1385(P-)Dfur::tet, NSA1385(P-) strain with a deletion of <i>fur</i> gene		This study
NewDisdD	NewDisdD::erm, <i>S. aureus</i> Newman strain with a deletion of <i>isdD</i> gene		This study
pNewDisd	pNewDisdD::erm, strain NewisdD::erm with the plasmid pLUG985 used for complementation		This study
IsdNewDisdD	IsdNewDisdD::erm, strain NewisdD::erm with the complementation of <i>isdD</i> gene (in pLUG985)		This study

DFU, diabetic foot ulcer

Quantitative Real Time RT-PCR for analysis of *isd* expression. Analysis of the mRNA levels of *isdA*, *isdB*, *isdD*, *fur*, *xerD*, and *sbi* was performed following the method previously described by Doumith et al. (Doumith et al, 2009) Amplifications were performed in triplicate from three different RNA preparations. Cycle threshold (Ct) values of the target genes were compared with the Ct values of the house-keeping *gyrB* gene, chosen as an endogenous reference for normalizing the transcription levels of the target gene. The Newman strain was used as control and the normalized relative expressions of the studied genes were determined for each strain according to the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{gene} - Ct_{gyrB})_{studied\ strain} - (Ct_{gene} - Ct_{gyrB})_{control\ strain}$. Primers used are listed in Table 1.

Siderophore production. An assay solution similar to that described by Schwyn and Neilands (Schwyn B and Neilands JB, 1987) was used to measure the siderophore production by the bacteria. A 0.021.9-g quantity of HDTMA was dissolved in 50 ml water in a 100 ml mixing cylinder. In a separate container 1.5 ml of 1 mM FeCl₃.6H₂O (in 10 mM HCl) was mixed with 7.5 ml of 2 mM CAS. This solution was slowly added to the HDTMA solution while stirring. The dissolved piperazine (4.307 g with 6.75 ml HCl to bring the pH to 5.6) was added to the mixture and the total volume was bringing to 100 ml. Finally, the CAS shuttle solution was obtained by adding 100 mg of 5-Sulfosalicylic acid. The CAS Shuttle solution was stored in the dark. The presence of siderophore will result in a reduction in blue color which can be measured at an absorbance of A₆₃₀ as loss of blue compared to a reference sample to the various desired time (30 min, 60 min, 90 min and 120 min). To measure the A₆₃₀ absorbance of sample we used the low iron LB medium as blank and the low iron LB medium plus CAS shuttle solution as reference sample. Results are calculated as a ratio between the absorbance of the sample (A_{630-S}) and the absorbance of the reference sample (A_{630-R}): $A_{630-S}/A_{630-R} = \% \text{ siderophore units}$ (Schwyn B and Neilands JB, 1987).

Results

The genetic island deregulates iron acquisition and promotes biofilm formation.

We observed an abnormal behaviour of colonization strains in iron depleted medium and we noted that the ROSA-like phage was inserted near the locus *isd*, the iron surface determinant system that transports heme-iron across the bacterial wall and membrane. We next sought to study the impact of the phage insertion on iron metabolism. In this regard, we analyzed the behaviour of six strains: the two clinical strains (NSA1385 and NSA739), the reference strain Newman (which harbors a different phage insertion at the Φ NM2 hotspot), a knockout mutant of the *isdD* gene in Newman strain (New Δ *isdD*), its complemented derivative pIsdNew Δ *isdD* and the control strain containing the whole plasmid (pNew Δ *isdD*). These last three strains allowed us to evaluate the impact of a mutation on iron metabolism in the *isd* locus (Table 2).

No difference could be detected in the growth curves of the different strains in LB medium while a slight reduction of growth of NSA1385 strain was seen in LB supplemented with iron (100 mM) (data not shown). However a significant difference was noted when bacteria were grown in LB-Chelex at 24h and

48h where no growth of the NSA1385 strain was observed unlike the other tested strains ($p<0.001$) (Figures 1A and 2).

The *isd* genes are normally induced by low iron concentrations. We used qRT-PCR to assess expression of *isdA*, *isdB*, *isdD* genes in bacteria grown in LB and LB-Chelex. Expression levels of these genes were all significantly ($p<0.001$) induced by low iron for all strains except for the colonizing NSA1385 strain (Figures 2 and 3). Expression of genes involved in iron uptake is controlled by the master repressor Fur. We used qRT-PCR to assess *fur* expression in the colonizing and infecting strains. Interestingly, while *fur* expression was repressed in low iron medium in the Newman and the infecting NSA739 strain, *fur* was highly induced in the colonizing NSA1385 strain ($p<0.001$) explaining the absence of *isd* gene expression (Figure 3). The qRT-PCR results show that the deregulation of iron metabolism is not due to the site of integration of the ROSA-like phage (since Newman, which behaved like the infecting strains, has Φ NM2 at the same location), but to a genetic element carried by the ROSA-like phage.

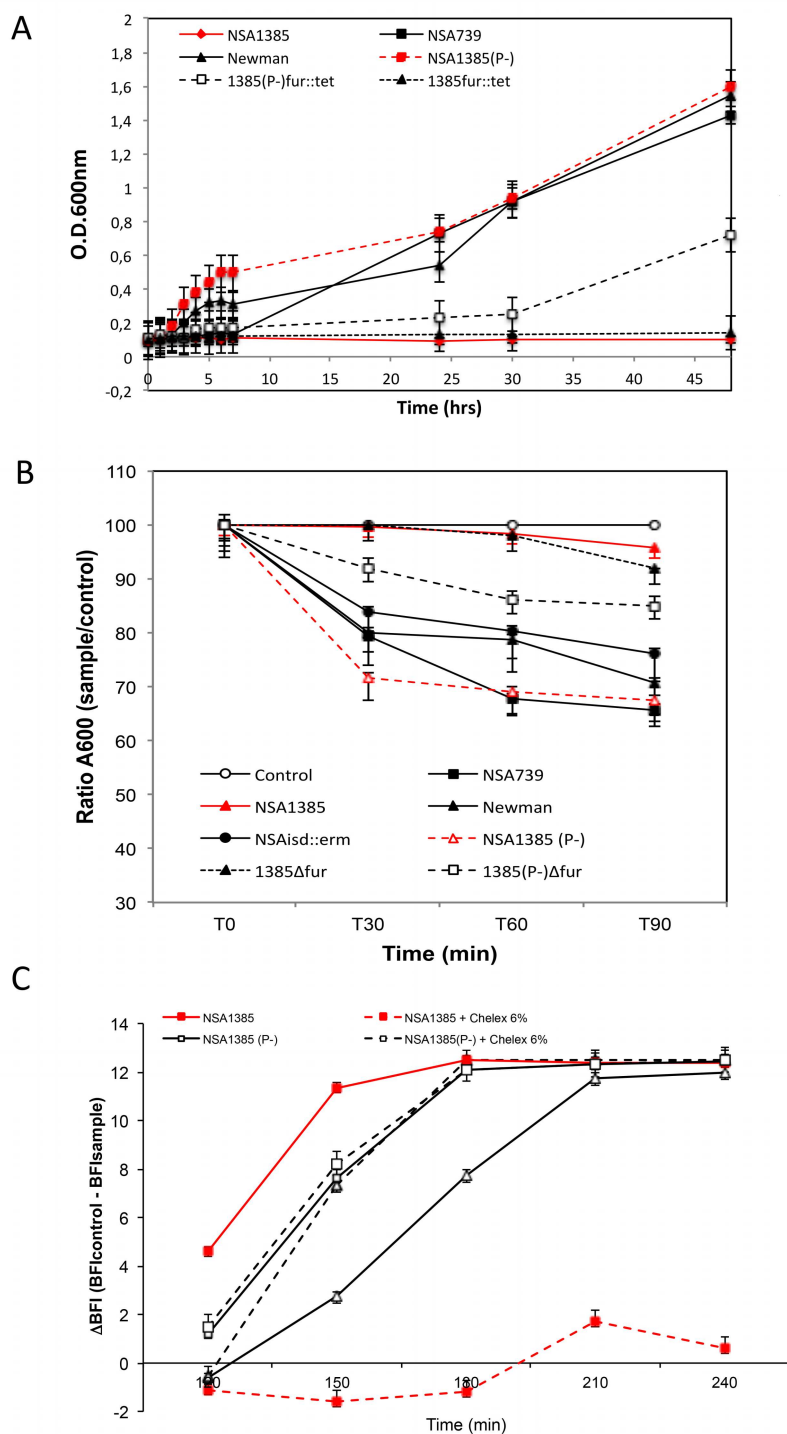


Figure 1. Growth curves, siderophore production and biofilm formation for the *S. aureus* strains.

A. Growth curves of the different *S. aureus* strains studied in presence of LB-Chelex.

B. Siderophore formation for the studied strains after 30, 60 and 90 min evaluated by chromazuril formation.

C. Kinetic of biofilm formation by the different *S. aureus* strains.

Bars represent the standard deviations from three independent experiments with triplicate for each one

Interestingly, while *fur* expression was repressed in low iron medium in the Newman and the infecting NSA739 strain, *fur* was highly induced in the colonizing NSA1385 strain ($p<0.001$) explaining the absence of *isd* gene expression (Figure 3). The qRT-PCR results show that the deregulation of iron metabolism is not due to the site of integration of the ROSA-like phage (since Newman, which behaved like the infecting strains, has Φ NM2 at the same location), but to a genetic element carried by the ROSA-like phage.

To determine the impact of this de-repression of *fur*, we evaluated siderophore production, a second way by which *S. aureus* can acquire iron in low iron medium. Using a chromazurol test, no variation in A_{600} (Absorbance 600 nm) was observed in NSA1385 strain cultured in LB-Chelex after 60 min and a slight decrease was observed after 90 min (p =not significant) (Figure 2B). In contrast, the other studied strains presented a significant decrease of the A_{600} after 30, 60 and 90 min in a LB-Chelex ($p<0.001$) (Figures 9B and 11). This result suggests that the colonizing NSA1385 strain does not produce siderophores, which contributes to the inability of this strain to grow in iron limited conditions.

Fur is also a regulator of biofilm formation (Johnson M et al, 2005). To determine the impact of the genetic insertion on Fur, we studied biofilm formation using the BioFilm Ring Test®. This assay is based on the immobilisation (or not) of magnetic beads embedded by bacterial aggregates or mats (Chavant P et al, 2007). The kinetics of biofilm formation with the Biofilm Ring Test® is shown in Figure 1C. The earliest times (Δ BFI (Biofilm Formation Index) <2) corresponds to complete bead mobility due to the absence of a biofilm. Increasing Δ BFI reflects the progressive immobilization of the beads during biofilm formation until reaching a plateau (>12) corresponding to complete immobilization of beads. In BHI medium, we observed that the colonizing NSA1385 strain formed biofilms more rapidly than the infecting NSA739 strain (150 min vs 180 min) (Figure 1C). Interestingly in a low iron medium (BHI-Chelex), the Δ BFI of the colonizing NSA1385 strain was under 2 after 240 min (Figure 1C) and stayed at this level after 24h and 48h. This result was not observed with the infecting NSA739 strain, which immobilized the beads more rapidly than the same strain cultured in BHI medium (a plateau reached at 180 min vs 210 min). These results confirmed the de-repression of *fur* in colonizing strains and the impact of this modification on iron metabolism and biofilm formation.

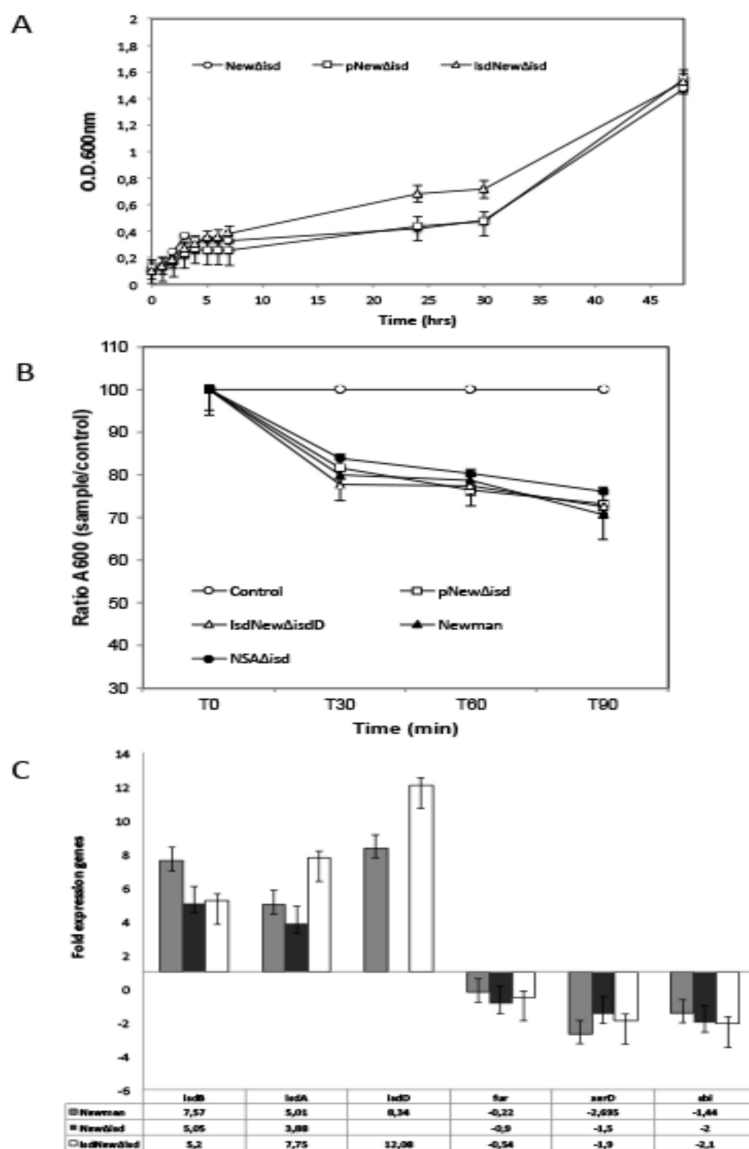


Figure 2. Growth curves, siderophore production and mRNA expression levels of different genes in low-iron conditions for the control *S. aureus* strains.

A. Growth curves of the different control *S. aureus* strains studied in presence of LB-Chelex.

B. Siderophore formation for the studied control strains after 30, 60 and 90 min evaluated by chromazurol formation.

C. Relative mRNA expression levels of different genes (*isdA*, *isdB*, *isdD*, *fur*, *xerD*, *sbi*) in low-iron conditions.

Mean and SD of genes expression relatively to that of strains in LB medium are indicated

Bars represent the standard deviations from three independent experiments with triplicate for

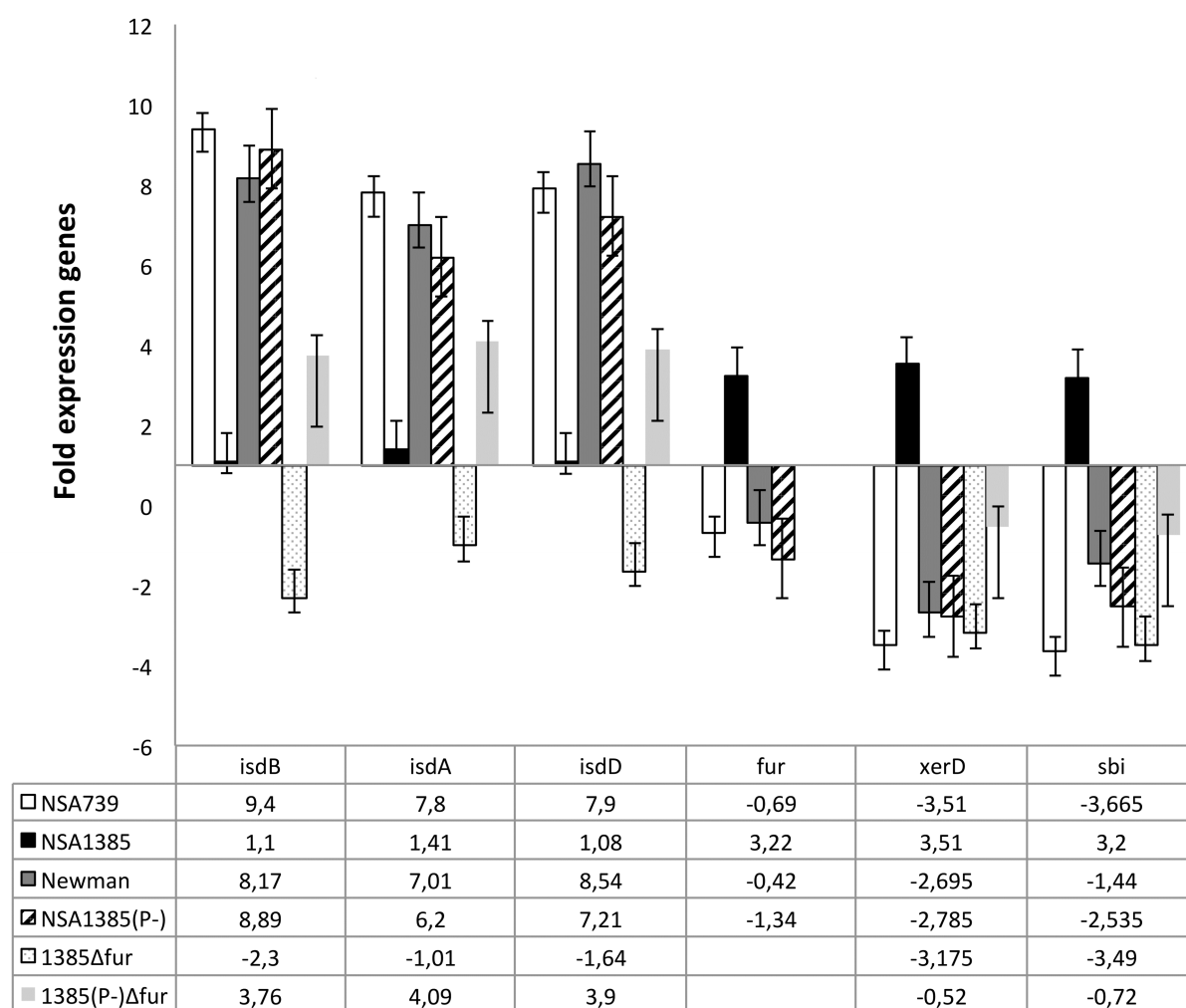


Figure 3. Relative mRNA expression levels of different genes in low-iron conditions.

Mean and SD of genes expression relatively to that of strains in LB medium are indicated.

The excision of the ROSA-like phage restores wild type iron metabolism.

To definitively understand the impact of the phage insertion in the colonizing strain, we evaluated the iron metabolism of NSA1385(P-), the strain obtained after excision of the ROSA-like phage (Table 2). We observed restoration of bacterial growth in low iron medium associated with the repression of *fur* and the increased expression of the *isd* genes (Figures 1A and 3). Moreover we noted the restoration of siderophore production (Figures 1B and 1C). All these data suggest that the ROSA-like phage clearly influences the virulence of the colonizing *S. aureus* strain notably by disrupting its iron metabolism by a de-repressing of the activity of the transcriptional regulator Fur.

The genetic island affects *S. aureus* virulence in a Fur-independent manner.

In order to establish how the ROSA-like insertion affects the iron uptake system, we investigated whether the Fur protein is active in addition to being transcribed in iron depleted condition. Fur is a protein that

uses iron as a cofactor becoming inactive in low iron environment. Therefore, we sought to determine whether this protein was active in its stringent condition. For this purpose, we evaluated the transcription of two genes positively regulated by Fur in iron-restricted conditions but not involved in *S. aureus* iron uptake: *xerD* (encoding a tyrosine recombinase), which is located in the same operon as *fur* and *sbi* (encoding a staphylococcal surface immunoglobulin-binding protein). We observed that in the colonizing NSA1385 strain both genes were induced in the iron depleted environment while they were repressed in the infecting strains (NSA739, Newman) and in NSA1385(P-) (Figure 3). These results suggest that the transcription of *xerD* and *sbi* genes is correlated with the transcription of *fur* in NSA1385 strain demonstrating that the Fur protein was active in iron-restricted conditions.

To establish a link between the low virulence of the NSA1385 strain and its inability to assimilate iron in a Fur-dependent manner, we constructed two mutants: a *fur* knock-out mutant of the NSA1385 (1385 Δ *fur*) strain and a *fur* knock-out mutant of the NSA1385(P-) (1385(P-) Δ *fur*) (Table 2). Firstly, we evaluated the metabolism of iron assimilation from these two strains by following their growth in a restricted iron environment. The 1385 Δ *fur* strain was unable to grow in iron-limited environment while 1385(P-) Δ *fur* showed only partially affected growth (Figure 1A). Secondly, we evaluated the transcription of the *isd* locus, the *xerD* and *sbi* genes as well as the production of siderophores. These two pathways are still affected in the 1385 Δ *fur* mutant even with the deletion of *fur* gene (Figure 3). However the expression of *xerD* and *sbi* was repressed confirming the absence of Fur production. The 1385(P) Δ *fur* strain partially restored the iron assimilation (Figures 1A and 3). Lastly, the *in vivo* virulence of these two strains showed that both strains were significantly less virulent than the infecting strain ($p < 0.001$). However the 1385 Δ *fur* strain was significantly less virulent than 1385(P-) Δ *fur* strain ($p < 0.001$) (Table 2). The deletion of *fur* was not sufficient to restore the virulence of the strain due to the central role played by Fur in virulence but also in other important metabolic pathways. These results show that the 1385 Δ *fur* strain retains a colonizing behaviour. We conclude that the genetic insertion affects the virulence and the iron assimilation system in a Fur-independent manner.

Travail n°6

Molecular epidemiology of *Staphylococcus aureus* strains isolated from inpatients with infected diabetic foot ulcers in an Algerian University Hospital.

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Aucune étude à ce jour n'avait évalué les caractéristiques des souches de *S. aureus* isolées de plaies du pied chez les patients diabétiques en Algérie. Le but de notre travail était de connaître l'épidémiologie moléculaire de souches de *S. aureus* isolées dans un CHU de l'est algérien et de comparer cette épidémiologie aux données françaises.

Des patients adultes diabétiques de type 1 et 2, ayant une plaie infectée du pied (Grades 2-4 ; SPILF 2007) ont été inclus du 1/04/2011 au 30/03/2012 au CHU d'Annaba (Algérie). Après préparation de la plaie, un prélèvement était réalisé soit par écouvillonnage pour les plaies superficielles, soit par ponction à l'aiguille fine ou biopsie osseuse pour les plaies profondes. Les prélèvements à *S. aureus* étaient conservés à température ambiante dans des géloses de conservation (BioRad) pour l'étude génotypique par des biopuces à ADN (StaphyType96®, Alere): détection de 330 gènes de virulence/résistance et assignation de l'origine clonale de la souche.

Durant l'étude, 85 souches de *S. aureus* ont été isolées chez 85 patients (62% d'hommes, âge médian : 64 ans (23-83)). 69 souches provenaient d'une plaie profonde et 16 d'ostéites. La prévalence des SARM était de 86% (n=73). De hauts niveaux de résistance associée étaient à noter pour les aminosides (89%), les quinolones (78%), le cotrimoxazole (67%) et la tétracycline (91%). Toutes les souches étaient sensibles aux glycopeptides. Parmi les SARM, la majorité (n=60, 82%) appartenait au clone brésilien ST239. 10 souches (14%) correspondaient au clone communautaire ST80. Parmi les souches sensibles à la méticilline, une plus grande diversité de clones a été détectée (7/12 souches) avec pour prédominance CC1 et CC15. De nombreux gènes de virulence étaient présents : hémolysines (*hlgv*, *hlgA*, *hla*) (100%), *cap8* (95%), entérotoxines (77%). Les gènes codant pour la toxine de Panton-Valentine (PVL) ont été détectés dans 12 souches (14%). Le suivi clinique des patients présentant ces souches a démontré de graves complications avec amputations dans les 12 cas.

Cette étude démontre la très forte prévalence des SARM et l'importance des souches PVL+ dans les plaies du pied chez les diabétiques en Algérie. Elle démontre la circulation d'un clone ST239 et la virulence de ST80. Ce travail permet d'apporter des informations nouvelles concernant l'épidémiologie algérienne très différente de celle rencontrée en France.

Molecular epidemiology of *Staphylococcus aureus* strains isolated from inpatients with infected diabetic foot ulcers in an Algerian University Hospital

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Abstract

Staphylococcus aureus is the most common pathogen cultured from diabetic foot infection (DFI). The consequence of its spread to soft tissue and bony structures is a major causal factor for lower-limb amputation. The objective of the study was to explore ecological data and epidemiological characteristics of *S. aureus* strains isolated from DFI in an Algerian hospital setting. Patients were included if they were admitted for DFI in the Department of Diabetology at the Annaba University Hospital from April 2011 to March 2012. Ulcers were classified according to the Infectious Diseases Society of America/International Working Group on the Diabetic Foot classification system. All *S. aureus* isolates were analysed. Using oligonucleotide arrays, *S. aureus* resistance and virulence genes were determined and each isolate was affiliated to a clonal complex. Among the 128 patients, 277 strains were isolated from 183 samples (1.51 isolate per sample). Aerobic Gram-negative bacilli were the most common isolated organisms (54.9% of all isolates). The study of ecological data highlighted the extremely high rate of multidrug-resistant organisms (MDROs) (58.5% of all isolates). The situation was especially striking for *S. aureus* [(85.9% were methicillin-resistant *S. aureus* (MRSA)), *Klebsiella pneumoniae* (83.8%) and *Escherichia coli* (60%)]. Among the *S. aureus* isolates, 82.2% of MRSA belonged to ST239, one of the most worldwide disseminated clones. Ten strains (13.7%) belonged to the European clone PVL+ ST80. *ermA*, *aacA-aphD*, *aphA*, *tetM*, *fosB*, *sek*, *seq*, *lukDE*, *fnbB*, *capB* and *agr* group I genes were significantly associated with MRSA strains ($p < 0.01$). The study shows for the first time the alarming prevalence of MDROs in DFI in Algeria.

Keywords: Algeria, diabetic foot ulcer, ecology, infection, multidrug-resistant organism, oligonucleotide array, *Staphylococcus aureus*

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Introduction

Diabetes mellitus is a serious public health problem that is rapidly expanding worldwide [1]. According to the National Institute of Public Health TAHINA survey, the prevalence of

diabetes was around 8% in Algeria in a population ranging from 35 to 70 years [2]. Foot ulcers are a common complication in diabetic patients, with prevalence as high as 15–25% [3]. These ulcers frequently become infected, and spread of infection to soft tissue and bony structures is a major causal factor for lower-limb amputation [4], making early diagnosis and adequate treatment essential. As microorganisms are normally present on skin wounds, diagnosis of infection must be based not on microbiological findings but on symptoms and clinical signs, as emphasized by the Infectious Diseases Society of America (IDSA), the International Working Group on the Diabetic Foot (IWGF) and the French Society for Infectious Pathology [4]. However, due to the confounding effect of neuropathy and ischaemia on local and systemic inflammatory responses, diagnosing diabetic foot infection (DFI) at an early

stage is often difficult, resulting in misuse of antibiotics [4]. Conversely, inappropriate antibiotic usage contributes to the increasing prevalence of multidrug-resistant organisms (MDROs), notably methicillin-resistant *S. aureus* (MRSA) [5–10]. To date very few epidemiological data are available about *S. aureus* strains isolated in diabetic foot ulcers (DFUs) and we are not aware of any such study that was performed in Algeria.

In this country, the European community-acquired MRSA clone ST80-IV harbouring Pantone–Valentine leukocidin (PVL) is responsible for more than one-third of both community and hospital infections [11,12]. Considering the high prevalence of diabetes mellitus in Algeria and the few data available about pathogens involved in DFI in this country, we decided to identify bacteria responsible for this condition; more specifically we aimed to determine the prevalence and the genotyping profiles of *S. aureus* from an Algerian hospital setting and to compare our data with those published in other countries. Finally, the identification of the role of MDROs in DFI might help clinicians to choose antibiotic agents in a more rational way.

Materials and Methods

Prospective study

Between 1 April 2011 and 30 March 2012, we prospectively enrolled all diabetic patients admitted to the Department of Endocrinology and Diabetology at Annaba University hospital (Algeria) for DFI. Patients were included if they had not received any antibiotic agents in the previous week. This study was carried out in accordance with the Declaration of Helsinki as revised in 2000. The presence and severity of DFI (grade 2–4) were assessed using the IDSA-IWGDF classification system [4].

After wound debridement, samples for bacterial culture were obtained by scraping the wound base and collecting debris by swabbing the wound base, needle aspiration or tissue biopsy and immediately sent to the bacteriology department.

Patients with PVL+ strains were monitored at 6 months to assess the wound outcome (healing/worsening).

Microbiological study

S. aureus was identified by conventional methods (Gram-positive cocci, catalase positive, mannitol fermenting, ability to coagulate rabbit plasma) (BioMérieux, Marcy-l'Étoile, France) and Pastorex Staph Plus agglutination (BioMérieux). Antimicrobial sensitivity was determined by the disk diffusion method according to recommendations of the Antibiotic Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org/pages/?page=746&id_page=182). Susceptibility to methicillin was

screened by agar diffusion using cefoxitin disks (BioRad, Marnes-La-Coquette, France). The *S. aureus* strains isolated during the inclusion period were preserved in deep agar gel. To be classified as a multidrug-resistant organism (MDRO), *S. aureus* should be methicillin resistant and Gram-negative bacilli resistant to the third generation cephalosporins (3GC). Those isolates included 3GC-resistant Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* resistant to ceftazidime.

Oligonucleotide DNA arrays and genotyping

Each *S. aureus* isolate collected during the study was analysed at the INSERM laboratory in Nîmes, France. The Alere StaphyType DNA microarray was used according to protocols and procedures previously detailed [13,14]. The test was performed in 5 h. The DNA microarray covers 333 target sequences, including species markers, SCCmec, capsule and *agr* group typing markers, resistance genes, exotoxins and MSCRAMM (microbial surface components recognizing adhesive matrix molecules) genes. Primer and probe sequences have been previously published [13]. The *S. aureus* strains were grown on Columbia blood agar and incubated overnight at 37°C. Culture material was enzymatically lysed prior to DNA preparation using commercially available spin columns (Qiagen, Hilden, Germany). Purified DNA samples were used as templates in a linear primer elongation with one primer per target. All targets were amplified simultaneously and, during this step, biotin-16-dUTP was incorporated into the resulting amplicons. Then amplicons were hybridized to the microarray, washed and blocked before addition of horseradish-peroxidase-streptavidin conjugate. After further incubation and washing steps, hybridizations were visualized as spots using a precipitating dye. A digital picture of the microarray was taken and analysed using a dedicated reader and software (ALERE Technologies, GmbH, Jena, Germany). The affiliation of isolates to clonal complexes (CCs) or sequence types (STs) as defined by MLST [14] was determined by an automated comparison of hybridization profiles with a collection of reference strains previously characterized [13,14]. A CC may be defined as a cluster of strains (clones) that are close enough together to be claimed to share to a common origin.

Statistical analysis

The presence of methicillin resistance in *S. aureus* strains was analysed according to demographic and clinical characteristics of study patients and the repartition of the different virulence genes using Fisher's exact test. Statistical analysis was performed using the S-Plus 2000 software package (Insightful Corporation, Seattle, WA, USA) and results were considered significant for $p < 0.05$.

Results

Clinical data and microbiological considerations

From April 2011 to March 2012, 128 patients were hospitalized for DFI and included in the study. Two hundred and seventy-seven strains were isolated (Table 1) from 183 samples, corresponding to a mean number of 1.51 isolates per sample. A polymicrobial infection was present in 92 samples (six with four bacteria, 17 with three bacteria, 69 with two germs). In 64 samples, infection was monomicrobial and 27 cultures were negative. Aerobic Gram-negative bacilli were the most commonly isolated microorganisms (54.9% of all isolates). Among them, Enterobacteriaceae were the most frequent (43.7% of all isolates), especially *Proteus* sp. (34.7% of enterobacteria). Non-fermentative Gram-negative bacilli were rather uncommon (11.2% of all isolates). Aerobic Gram-positive cocci accounted for 45.1% of all organisms, with *S. aureus* as the most commonly isolated pathogen ($n = 85$, i.e. 30.7% of the aerobic bacteria and 68% of the Gram-positive cocci). Surprisingly, *Streptococcus* sp. was very rarely detected (0.7% of all isolates). No anaerobes were isolated. A high number of isolates were MDRO (58.5%). MRSA was the most common resistant bacteria (85.9% of *S. aureus*, 45.1% of all resistant isolates). Among Gram-negative bacilli, 57% of enterobacteria were multidrug resistant, with a dramatically high percentage of *Klebsiella pneumoniae* (83.8%) and *Escherichia coli* (60%). Moreover all *Acinetobacter baumannii* and more than half of *P. aeruginosa* were MDRO.

As previously mentioned, *S. aureus* was isolated in 85 patients from the bacterial culture of their infected wound. The demographic and clinical characteristics of these patients

are shown in Table 2. Twenty-one DFIs (24.7%) were classified as grade 2, 55 as grade 3 (64.7%) and nine as grade 4 (10.6%). In 40 patients (47.1%) the current wound was the first episode of ulceration. Sixteen wounds (18.8%) were associated with osteomyelitis. There was no significant difference in the baseline characteristics of patients regarding whether their wounds were infected by methicillin-sensitive *S. aureus* (MSSA) or MRSA, except that previous antibiotic treatment was significantly more frequent in patients with MRSA strains ($p = 0.007$) (Table 2).

Antimicrobial susceptibility of *S. aureus*

The *in vitro* activities of antimicrobial agents against the 85 *S. aureus* isolates demonstrated a high level of resistance to all classical antistaphylococcal treatments.

All MSSA isolates were susceptible to pristinamycin, fosfomycin and glycopeptides. Gentamicin was the most active aminoglycoside (41.6%). Regarding MRSA, only glycopeptides were active against all the isolates. Compared with MSSA, a significantly higher percentage of MRSA was resistant to some antimicrobial agents, including aminoglycosides, quinolones, cotrimoxazole and tetracycline ($p < 0.01$).

Clonal complexes (CCs) distribution of *S. aureus*

Using the DNA arrays technology, all the *S. aureus* strains we isolated were analysed. The majority of the 73 MRSA belonged to Brazilian clone ST239 ($n = 60$, 82.2%). Ten strains (13.7%) were assigned to the European clone ST80 and the last three (4.1%) to a CC unknown in the database.

Regarding MSSA, they belonged to a great diversity of CCs: CCI ($n = 3$), CCI5 ($n = 3$), CCI21 ($n = 2$), CC9 ($n = 1$), CC54 ($n = 1$) and ST152 ($n = 1$). One isolate was contained in a CC unknown in the database. Concerning the strains isolated from bone, all were MRSA; 14 belonged to ST239 and two to clone ST80.

Resistance and virulence profiles

The prevalence of resistance and virulence determinants are summarized in Table 3.

Analysis of the resistance genes completely agreed with conventional susceptibility data. All MRSA isolates were detected by the cefoxitin test and were positive for *mecA* and *SCCmec* cassette by DNA arrays. The most prevalent macrolide resistance gene in MRSA strains was *ermA*, which was detected in 39.7% ($n = 29$) of the isolates, whereas *ermC* was found as a single *erm* gene in 16.6% ($n = 2$) of MSSA isolates. The aminoglycoside resistance genes were found only in MRSA strains; *aphA* and *aacA-aphD* were the most prevalent genes (95.9% and 90.4% of the isolates, respectively). The tetracycline resistance genes (*tet* efflux genes) were detected

TABLE 1. Bacteriological aetiology of diabetic foot infections

Microorganism(s)	N (%)	Multidrug-resistant bacteria* (%)
Gram-positive aerobic cocci	125 (45.1)	
<i>Staphylococcus aureus</i>	85 (30.7)	73 (85.9)
<i>Enterococcus faecalis</i>	7 (2.5)	—
Coagulase-negative <i>Staphylococcus</i>	31 (11.2)	—
<i>Streptococcus</i> spp.	2 (0.7)	—
Gram-negative aerobic bacilli	152 (54.9)	
<i>Proteus mirabilis</i>	35 (12.6)	12 (34.3)
<i>Klebsiella pneumoniae</i>	31 (11.2)	26 (83.8)
<i>Escherichia coli</i>	20 (7.2)	12 (60)
<i>Morganella morganii</i>	15 (5.4)	8 (53.3)
<i>Enterobacter cloacae</i>	9 (3.2)	5 (55.5)
<i>Proteus vulgaris</i>	7 (2.5)	4 (57.1)
<i>Providencia stuartii</i>	2 (0.7)	1 (50)
<i>Klebsiella oxytoca</i>	1 (0.4)	1 (100)
<i>Citrobacter</i> spp.	1 (0.4)	—
<i>Pseudomonas aeruginosa</i>	23 (8.3)	12 (52.1)
<i>Acinetobacter baumannii</i>	8 (2.9)	8 (100)

*Multidrug-resistant bacteria included methicillin-resistant *S. aureus*, Enterobacteriaceae resistant to third-generation cephalosporin, *P. aeruginosa* or *Acinetobacter baumannii* resistant to ceftazidime.

TABLE 2. Demographic and clinical characteristics of study patients

Characteristics	Value ^a			P MSSA vs. MRSA
	Patients with MSSA n = 12	Patients with MRSA n = 73	Total n = 85	
Age (range), years	63 (47–82)	64.5 (23–83)	64 (23–83)	NS
Male/female, n (%)	7 (58.3) / 5 (41.7)	46 (63)/27 (37)	53 (62.3)/32 (37.7)	NS
Type 1/Type 2 diabetes mellitus	2/10	16/57	18/67	
Cardiovascular disease				
Absence	4 (33.3)	18 (24.6)	22 (25.9)	NS
Coronary heart disease	0 (0)	8 (11)	8 (9.4)	NS
Peripheral arterial disease	7 (58.3)	46 (63)	53 (62.3)	NS
Arterial hypertension	9 (75)	43 (58.9)	52 (61.1)	NS
Stroke	1 (8.3)	2 (2.7)	3 (3.5)	NS
Nephropathy				
Absence	7 (58.3)	32 (43.8)	39 (45.9)	NS
Microalbuminuria	1 (8.3)	13 (17.8)	14 (16.4)	NS
Proteinuria	2 (16.7)	17 (23.3)	19 (22.3)	NS
Renal failure	3 (25)	15 (20.5)	18 (21.2)	NS
Neuropathy				
Peripheral	10 (83.3)	56 (76.7)	66 (77.6)	NS
Autonomic	0 (0)	5 (6.8)	5 (5.9)	NS
Diabetic retinopathy				
Absence	5 (41.7)	21 (28.7)	26 (30.6)	NS
Non-proliferative diabetic retinopathy	5 (41.7)	41 (56.1)	46 (54.1)	NS
Proliferative diabetic retinopathy	2 (16.7)	11 (15.1)	13 (15.3)	NS
Lifestyle factors				
Obesity	5 (41.7)	15 (20.5)	20 (23.5)	NS
Smoking	2 (16.7)	13 (17.8)	15 (17.6)	NS
Alcoholism	1 (8.3)	2 (2.7)	3 (3.5)	NS
Sedentary	6 (50)	23 (31.5)	29 (34.1)	NS
First wound/ recurrence	7 (58.3)/5 (41.7)	33 (45.2)/40 (54.8)	40 (47.1)/45 (52.9)	NS
Prior antibiotic therapy	8 (66.7)	70 (95.9)	78 (91.7)	0.007
β-Lactams	6 (50)	53 (72.6)	59 (69.4)	NS
Aminoglycosides	1 (8.3)	7 (9.6)	8 (9.4)	NS
Quinolones	2 (16.7)	9 (12.3)	11 (12.9)	NS
Macrolides	0 (0)	12 (16.4)	12 (14.1)	NS
IDSA grade				
2	4 (33.3)	17 (23.3)	21 (24.7)	NS
3	8 (66.7)	47 (64.4)	55 (64.7)	NS
4	0 (0)	9 (12.3)	9 (10.6)	NS
Samples				
Scraping-swabbing	12 (100)	63 (86.3)	75 (88.2)	NS
Needle aspiration	0 (0)	6 (8.2)	6 (7.1)	NS
Tissue/bone biopsy	0 (0)	4 (5.5)	4 (4.7)	NS

NS, not significant.

^aValues are numbers and percentages in brackets.

in 98.6% ($n = 84$) of the isolates. Compared with MSSA, MRSA strains exhibited a significantly higher prevalence rate for *ermA*, *aacA-aphD*, *aphA*, *sat*, *tetM* and *fosB* genes ($p < 0.01$). No *van* genes were detected, in agreement with the *in vitro* susceptibility data.

Strains were characterized by some distinctive features regarding the distribution of virulence factors: high prevalence of enterotoxins (*sek* ($n = 65$, 76.5%), *seq* ($n = 64$, 75.3%) and *sea* ($n = 38$, 44.7%)), haemolysins (*hlg*, *hlgv*, *hlgA*, *hld*), genes encoding intracellular adhesion proteins (*icaA*, *icaC*, *icaD*) and capsular polysaccharide type 8 ($n = 81$, 95.3%), together with four genes encoding MSCRAMM (*ebpS*, *clfA*, *clfB*, *fmbA*). A majority of the isolates were found in *agr* group 1 ($n = 64$, 75.3%). *seb*, *egc* cluster, *seh*, *hla*, *cap5* and *agr* group 2 were significantly more prevalent in MSSA strains and *sek*, *seq*, *lukDE*, *fmbB*, *cap8*, and *agr* group 1 in MRSA strains ($p < 0.01$). Genes encoding exfoliatin A and B toxins were not found. On the other hand, *etD* was detected in ten (11.8%) strains. DNA array analysis revealed that 12.9% ($n = 11$) of isolates were *edinB* positive. In ten strains, *edinB* and *etD* genes were found to be associated. Genes encoding PVL were detected in 11

MRSA isolates (15%) and in one MSSA isolate (8.3%). All these strains belonged to the CC80-MRSA (European community MRSA Clone). A 6-month follow-up of the patients harbouring these strains demonstrated that all of them had a poor outcome (amputations).

Discussion

This study highlighted the extremely high rate of MDRO (58.5%) in DFI from Algerian patients. The prevalence of MDRO was especially striking for *S. aureus* (85.9% of MRSA among staphylococci), *Klebsiella pneumoniae* (83.8%) and *Escherichia coli* (60%). This burden of resistance might be directly associated with DFI because a national survey highlighted that the MRSA rate is 35% in Algeria, increasing to 40.7% for hospitalized patients (www.sante.dz/aarn/documents/pdf/rapportII.pdf). The same trend was found for *K. pneumoniae* (52.5% were resistant to cefotaxime) and *E. coli* (13.4%). Another surprising feature was the predominance of Gram-positive cocci as *S. aureus* is considered to be the most

TABLE 3. Main virulence profiles and resistance determinants of *S. aureus* isolated from diabetic foot ulcers

	MSSA n = 12	MRSA n = 73	TOTAL n = 85	p MSSA vs. MRSA
Virulence genotyping				
Enterotoxins				
sea	4 (33.3)	34 (46.5)	38 (44.7)	NS
seb	3 (25)	0 (0)	3 (3.5)	0.002
egc cluster	5 (41.6)	2 (2.7)	7 (8.2)	<0.001
seh	3 (25)	0 (0)	3 (3.5)	0.002
sek	3 (25)	62 (85)	65 (76.5)	<0.001
seq	3 (25)	61 (83.5)	64 (75.3)	<0.001
Other toxins				
tst	0 (0)	0 (0)	0 (0)	NS
etA	0 (0)	0 (0)	0 (0)	NS
etB	0 (0)	0 (0)	0 (0)	NS
etD	0 (0)	10 (13.7)	10 (11.8)	NS
edinA	0 (0)	0 (0)	0 (0)	NS
edinB	1 (8.3)	10 (13.7)	11 (12.9)	NS
lukS-PV/lukF-PV	1 (8.3)	11 (15)	12 (14.1)	NS
lukDE	9 (75)	73 (100)	82 (96.5)	0.02
Haemolysins				
hla	12 (100)	12 (16.4)	24 (28.2)	<0.001
hld	12 (100)	73 (100)	85 (100)	NS
hlgA	12 (100)	73 (100)	85 (100)	NS
hlg	12 (100)	73 (100)	85 (100)	NS
hlgv	12 (100)	73 (100)	85 (100)	NS
MSCRAMM				
bbp	12 (100)	72 (98.6)	84 (98.8)	NS
cna	7 (58.3)	62 (84.9)	69 (81.2)	NS
ebpS	12 (100)	73 (100)	85 (100)	NS
clfA	12 (100)	73 (100)	85 (100)	NS
clfB	12 (100)	73 (100)	85 (100)	NS
fib	10 (83.3)	73 (100)	83 (97.6)	NS
fmbA	12 (100)	73 (100)	85 (100)	NS
fmbB	0 (0)	60 (82.2)	60 (70.6)	<0.001
Capsules				
cap5	3 (25)	1 (1.3)	4 (4.7)	0.008
cap8	9 (75)	72 (98.6)	81 (95.3)	0.008
Other virulent factors				
chp	6 (50)	33 (45.2)	39 (45.9)	NS
scn	12 (100)	72 (98.6)	84 (98.8)	NS
Accessory gene regulator				
agr1	2 (1.6)	62 (84.9)	64 (75.3)	<0.001
agr2	5 (41.6)	2 (2.7)	7 (8.2)	<0.001
agr3	3 (25)	10 (13.7)	13 (15.3)	NS
agr4	3 (25)	16 (21.9)	19 (22.4)	NS
Resistance genes				
mecA	0 (0)	73 (100)	73 (85.9)	<0.001
ermA	0 (0)	29 (39.7)	29 (34.1)	0.004
ermC	2 (16.7)	1 (1.3)	3 (3.5)	0.05
aacA-aphD	0 (0)	66 (90.4)	66 (77.6)	<0.001
aphA	0 (0)	70 (95.9)	70 (82.4)	<0.001
sat	0 (0)	70 (95.9)	70 (82.4)	<0.001
tetM	0 (0)	63 (86.3)	63 (74.1)	<0.001
tet efflux	12 (100)	72 (98.6)	84 (98.8)	NS
fosB	8 (66.6)	64 (100)	72 (84.7)	0.03

NS, not significant.

*Values are numbers and percentages in brackets.

frequently isolated and virulent pathogen in DFI from western countries [15–17]. However, some recent studies have shown that Gram-negative organisms are the most frequent isolates in DFI from patients living in warm climates, especially in India [18–20]. As noted by Lipsky *et al.* [4], there is no clear explanation for this predominance.

The increasing prevalence of antibiotic-resistant bacteria in DFUs, particularly MRSA, both as colonizers or pathogens [6–8], is problematic. MRSA has emerged as a serious and commonly occurring problem in diabetic patients with foot ulcers [5,7]. MRSA requires targeted antibiotic treatment and has been associated with a poor outcome in many reports [9,10,21]. In this study, we focused for the first time on *S. aureus* isolated from DFIs in Annaba University Hospital

(Algeria). Even though Gram-negative bacilli as a group were the most prevalent bacteria isolated in our study, *S. aureus* was the most common isolate (Table 1), accounting for 30.7% of all microorganisms, and 85.9% of *S. aureus* were methicillin resistant, in agreement with other studies [5,15,17,22,23]. However, the rate of methicillin resistance is particularly high, ranging from 15 to 30% around the world [24]. While the MRSA prevalence rate is clearly high in Annaba Hospital (62.5% of all *S. aureus* isolated in 2011 were MRSA), it is significantly lower than in strains isolated from DFIs ($p < 0.001$). This high prevalence rate is associated with previous antibiotic treatment in our study and is likely to be attributable to the high proportion of patients with recurrent ulcers (55%): all were previously infected and had been already treated with antibiotics during a preceding hospitalization, a well-established risk factor for selecting antibiotic-resistant organisms, especially in a diabetic foot clinic [3,4,8]. β -Lactams were the most prescribed antibiotic treatment in this population. Potential sources of acquisition of MRSA may include prior hospitalizations and transmission by healthcare providers at home during dressing changes. Even if MRSA is considered to be a colonizer, we could observe in this population that all the osteomyelitis was due to MRSA strains, suggesting that if a rapid debridement is not performed all the strains are a source of serious complications in chronic wounds.

DNA microarray is a technique from molecular biology that is able to detect genes related to virulence and antibiotic resistance [13,14]. Using this method, we were able to compare the strains, determine their clonality and evaluate the virulence and resistance profiles of *S. aureus* [13,14]. In our study, 82.2% of the MRSA isolates we tested belonged to ST239; this clone is considered to be one of the most worldwide disseminated clones [25], being extensively described as a major clone in many countries in Asia, Europe, South America [26] and Africa [27]. It has not been described in Algeria to date. Interestingly, in this study we highlighted the importance of strains harbouring Pantone–Valentine leukocidin-encoding genes ($n = 12$, 14.1%). These genes coding for a cytotoxin are claimed to be a major threat in severe tissue necrosis [28] in some but not all previously published studies. This high prevalence is unknown in this pathology, where these strains are rarely isolated from chronic wounds [22,23] and their pathogenicity is low in this setting [29]. In this study, all PVL+ MRSA strains belonged to CC80; this clone has been considered to be the main clone associated with PVL in Europe [30], and also in Algeria [11] and Tunisia [31]. The high prevalence of this clone in Algeria could explain its detection in DFI. However, more surprisingly, all the patients harbouring PVL+ strains had a worse outcome, a result not found in a previous study [29]. Differences in wound care and treatment

compliance or indications for lower-limb amputations could explain these differences.

One of the limitations of the study is the high number of swab samples (93%) but these samples were obtained after scraping the base of the ulcers, as recommended by the IWGDF [4]. Moreover, the number of isolates per sample was low, suggesting that the sampling method was rather inadequate [32].

In conclusion, this study highlighted for the first time the bacterial ecology of DFI in an Algerian hospital, showing a high prevalence of MDRO, notably MRSA strains and a high proportion of Gram-negative bacilli. These features appear close to those previously described in India [18–20]. This trend is alarming and could indicate a misuse of antibiotic agents and/or high prevalence of cross-transmission of microorganisms. Improving knowledge of the local ecology of DFI in Annaba looks to be important for a more rational empirical prescription of antibiotic agents in Algeria if such results are confirmed by further studies in other Algerian areas.

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Transparency Declaration

Nothing to declare.

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Autre Travail

Mass spectrometry: a revolution in clinical microbiology?

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Review

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Mass spectrometry: a revolution in clinical microbiology?

Abstract: Recently, different bacteriological laboratory interventions that decrease reporting time have been developed. These promising new broad-based techniques have merit, based on their ability to identify rapidly many bacteria, organisms difficult to grow or newly emerging strains, as well as their capacity to track disease transmission. The benefit of rapid reporting of identification and/or resistance of bacteria can greatly impact patient outcomes, with an improvement in the use of antibiotics, in the reduction of the emergence of multidrug resistant bacteria and in mortality rates. Different techniques revolve around mass spectrometry (MS) technology: matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), PCR combined with electrospray ionization-mass spectrometry (PCR/ESI-MS), iPLEX MassArray system and other new evolutions combining different techniques. This report emphasizes the (r) evolution of these technologies in clinical microbiology.

Keywords: clinical microbiology; iPLEX MassArray; MALDI-TOF MS; mass spectrometry; PCR/ESI-MS.

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Introduction

Despite the extensive use of antibiotics and vaccination programs, infectious diseases, particularly bacterial infections, remain a major cause of morbidity and mortality worldwide. One of the great challenges of microbiology for the coming years remains the development of new antimicrobial agents. Indeed, because of the massive and often untimely use of antibiotics, pathogenic bacteria have developed resistance mechanisms against most classes of antibiotics currently available. This trend has been particularly dramatic over the past 20 years. The possible transmission of genes encoding mechanisms of resistance between different bacterial species has led to the emergence, particularly in the hospital, of multi-drug and pain resistant bacteria that led to increasing difficulties in therapeutic management. This major public health problem also faces a challenging reality: the virtual withdrawal of the pharmaceutical industry from the development of new antibiotics. No blockbuster drug is being promoted [1, 2]. Given this reality, the development of new therapeutic strategies should be considered. One strategy is the best use of antibiotics. In this way the recent (r)evolution in clinical microbiology approach could help to improve this problem. Different solutions have increased: first the molecular diagnostic methods (e.g., 16S ribosomal RNA sequencing, real-time PCR for detection of selected genes) which is classically used in parallel with routine bacteriological methods; and second mass spectrometry (MS) which is propelling us into a new era far beyond the classical bacteriology of Louis Pasteur. These promising new broad-based techniques have merit, since they can rapidly identify many bacteria, including organisms that are difficult to culture or new emerging strains. They also can be used as epidemiological tools to follow disease transmission. The benefit of rapid reporting of isolation and/or identification of resistance of bacteria can potentially impact patient outcome, improve the use of antibiotics and

reduce the emergence of multidrug resistant bacteria and mortality rates.

The aim of this review is to describe the different solutions using MS recently developed and to evaluate their impact on rapidity of diagnosis and on the prognosis of infectious diseases.

The MALDI-TOF MS technology

Principle (Figure 1)

The first description concerning the use of MS in bacteriology was in 1975 [3]. The technology was developed to study the biomarker profiles of some bacterial species. It used the ionization by fast atom bombardment and the association of gas chromatography and MS. The difficulty was to detect the release of the ribosomal and membrane proteins without destroying them in order to analyze the protein profiles and obtain mass pattern spectra. This encouraging study was not followed by any other development until 1996. At this time, the first matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) experiment was successful in identifying bacteria directly from whole colonies based on protein content [4, 5]. However, the most important evolution corresponded to the system of detection by soft ionization techniques such as MALDI and electrospray ionization (ESI) allowing the analysis of biomolecules and large organic molecules, which tend to be fragile when ionized by the old other conventional ionization methods [6]. The continual development of the hardware provided increasing accuracy and resolution of the different proteins, and the MALDI-TOF MS was being used for the identification of bacteria in research settings [4]. Following this period, the new approaches for species identification were developed involving the use of a different matrix. The change of matrix allowed the ionization of mainly ribosomal proteins, which are more conserved than surface proteins [7]. This was considered to be more reliable for routine identification of bacterial species, as culture conditions seemed to have little effect on the results of identification [8]. Therefore, the MS became a revolutionary tool for bacteriology laboratories.

Matrix-assisted laser desorption/ionization involves the principle of the co-crystallization of the sample with a matrix. The couple sample-matrix is irradiated with photons of a laser whose wavelength is in the absorption band of the matrix. This radiation causes the ionization in the gas phase of molecules of the sample and matrix

(Figure 1). The ions formed are then accelerated and sent in a vacuum flight tube where they are separated according to their speed. This speed depends itself of a mass/charge ratio peak (m/z). Ions characterized by a high m/z fly more slowly than those with lower m/z . The approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. Proteins of different amino acid sequence produce a series of peptides masses, which can be detected by the detector. The spectrum of identified peptide masses is unique for a protein specific to a bacterial species. The peptide profiles are generated from direct ionization of an intact colony or a bacterial protein extract after manual extraction. The spectrum obtained is then compared with spectra contained in the database according to the algorithm-specific software used. Identification occurs after a protein's spectral signature is correlated to a database of spectra collected from reference strains. The results are returned with a scoring system, which appears to be conservative enough to avoid false-positive identifications with both systems [9, 10]. Different systems (software/database) have been created for routine identification of bacteria: the Bruker instrument provides its own solution, MALDI BioTyper (software, bioinformatic and database); the Shimadzu instrument uses also its own software (Launchpad) and the SARAMIS database developed by AnagnosTec GmbH and recently acquired by BioMérieux; Andromas (a French start-up) provides a different type of database and software for routine bacteriology, compatible with either Bruker or Shimadzu hardware. These databases currently available for both systems need to be optimized for certain species but are large and contain up to 2000 species (including bacteria, yeast and mycobacteria), with over 3000 spectra. Their performance is in any case higher than phenotypic identification systems [11–13]. We could note that no statistically significant difference was identified between the platforms for clinically relevant bacteria [11, 14].

As we previously noted, MS needs the use of a matrix to facilitate the ionization of proteins. They allow a burst of microorganisms and the release of proteins that migrate performing a true chromatography. Depending on the matrix, we obtain a spectrum of proteins of specific molecular weight ranges. Alpha-4-cyano-4-hydroxycinnamic acid (HCCA) induces the formation of small spherical crystals with more uniform distribution. It is not suitable for some taxons and requires fewer laser shots and allows the production of 80 to 150 peaks per spectrum [15]. The UV absorbing matrices used were found to be highly specific to bacterial Gram type: HCCA for Gram-negative bacteria and

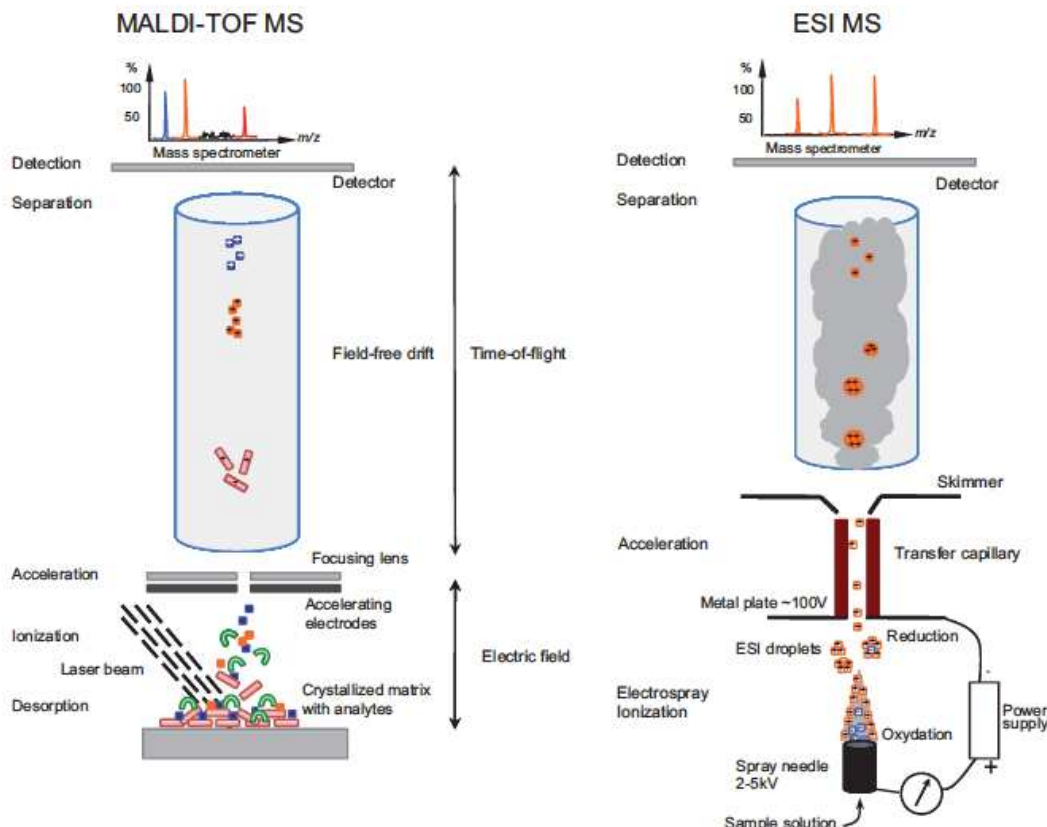


Figure 1 Principle of MALDI-TOF MS and ESI-MS identification of bacteria.

For MALDI-TOF, laser impact causes thermal desorption of ribosomal proteins of bacteria embedded in matrix material and applied to the target plate (analytes shown as red, light blue, and orange spheres, the matrix is given as green spheres). In an electric field, ions are accelerated according to their mass and electric charge. The drift path allows further separation and leads to measurable differences in time-of-flight of the desorbed particles that are detected on top of the vacuum tube. From the time-of-flight, the exact mass of the polypeptides can be calculated. For ESI, the DNA amplicons are dissolved in a solvent and injected in a conductive capillary, where high voltage is applied, resulting in the emission of aerosols of charged droplets of the sample. The latter are sprayed through compartments with diminishing pressure, resulting in the formation of gas-phase multiple-charged analyte ions, which then are detected by spectrometer.

5-chloro-2-mercaptobenzothiazole for Gram-positive bacteria [16]. The latter matrix system enhances the sensitivity of the analysis of bacterial endotoxins (lipid A) by more than 100-fold and provides tolerance to high concentrations of reagents (such as sodium dodecyl sulphate, sodium chloride and calcium chloride) [17]. The 2,5-dihydroxybenzoic acid (DHB) allows the formation of long crystals from the periphery to the center of the deposit. It is suitable for a majority of taxons and requires more laser shots to obtain 100 to 200 peaks per spectrum and many signals whose the m/z is >10 kDa [18]. The manipulation of this matrix is trickier. Finally 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid or SA) is a more recent matrix. It allows the analysis of

proteins of higher molecular weight than the HCCA and DHB [19].

Advantages of MALDI-TOF MS (Table 1)

The MALDI-TOF MS is the most promising technology for the present and the future in the microbiology laboratories. This is due to its ability to analyze whole bacterial cells with virtually no sample preparation or no batching and the improvement in the identification time of a positive culture (10–20 s for acquisition of the protein spectra and 15–30 s for the comparison in the databank), starting from a colony (Figure 2).

	MALDI-TOF	PCR/ESI-MS	IPLEX Mass array	SELDI TOF	LC-ESI-QqQ-MS
Interest	Excellent specificity High throughput: detection of multiple spots [20–67] in one experiment Bacterial detection from urine sample Adapted to bacteriology lab workflow	Excellent sensitivity and specificity Powerful tool for epidemiological investigation	Excellent sensitivity and specificity High throughput: analysis of 96 or 384 samples on a same chip Powerful tool for phylogenetic and epidemiological investigation	High throughput Minimal sample volume required Directly from crude biological samples Peptide sensitivity in the femtomolar range	Excellent specificity (higher than MALDI)
Limits	Requires often culture Limited to bacterial genera and species, more difficult for sub-species Quantification detection difficult Very easy 1–2 min after culture (18 h) Proteins, glycopeptides, oligonucleotides, carbohydrates	Batching of six samples at a time Need to DNA extraction (which increases the costs) Need a validation Moderate 4–6 h without culture PCR amplicons	Cost of the equipment	Not link to MS (no peptide identification) Low mass resolution Low mass accuracy	Research tool No bacterial application Not adapted for lab workflow Cost of the equipment High Min to h Molecules, peptides
Complexity of data analysis	Very easy	Need a validation	High	High	High
Time to result	1–2 min after culture (18 h)	4–6 h without culture	Few seconds after PCR (4 h)	Min to h	Min to h
Molecules detected	Proteins, glycopeptides, oligonucleotides, carbohydrates	PCR amplicons	PCR amplicons	Native proteins, peptides	Molecules, peptides
Mono/polymicrobial detection	Mono microbial	3–4 bacteria	Polymicrobial	Polymicrobial	Polymicrobial
Detection of new organism	Possible but limited	Yes and also virus	Yes and families of virus	Yes	Yes
Running costs	Low	High	High	High	Low

Table 1 Comparison between the different technologies using mass spectrometry in bacteriology.

MALDI-TOF MS is also widely used because of its high accuracy, low running cost and low maintenance needs. Indeed this technology requires only the medium to grow the organism and a small quantity of matrix. It has already replaced most of the biochemical tests currently used for bacterial identification in routine laboratories (e.g., catalase, oxydase, identification card or API gallery, latex test, agglutination tests) because it does not require prior knowledge about the organism [68]. This new method has now proven to be reliable and safe for the identification of the clinically relevant bacteria (e.g., *Enterobacteriaceae*, the non-fermenting bacteria, staphylococci or streptococci) [12, 69–72]. Continuous improvement of the database is performed and updates are released every 3–6 months. In this way, the identification of most rarely isolated bacteria such as some potential bioterrorism agents (*Brucella* sp. [73], *Coxiella burnetii*, *Francisella tularensis* and *Bacillus anthracis* [74]), some Gram-negative bacilli (*Pasteurellaceae* [75], *Acinetobacter baumannii* group [76, 77], *Yersinia* sp. [78], *Legionella* sp. [79]), anaerobes [80–82], or bacteria difficult to identify after Gram staining (*Leptospira* sp. [83], *Mycobacterium* sp. [84, 85]) have been reported.

All these developments are promising notably since the publication by Gaillot et al. on cost-effectiveness of MS. The authors reported that phasing out of conventional techniques in favor of MS resulted in the overall saving of \$177,090 in 1 year [86].

Inconveniences of MALDI-TOF MS (Table 1)

The speedy identification of bacteria by MS clearly presents a major advantage for clinicians in the management of antibiotic treatment. However, even if the protein mass pattern spectra can be analyzed for identification of bacteria to the genus and species level, many results are rarely available to the subspecies level. This means that this tool is not completely efficient to identify all bacteria (e.g., difficulty in distinguishing between *Escherichia coli* and *Shigella* sp.) and does not provide help in epidemiological studies to follow crossed transmission of bacteria. Moreover, the major problem is the incomplete current databases. The composition and quality of these databases is crucial for a correct identification. They still need implementation and expansion [68].

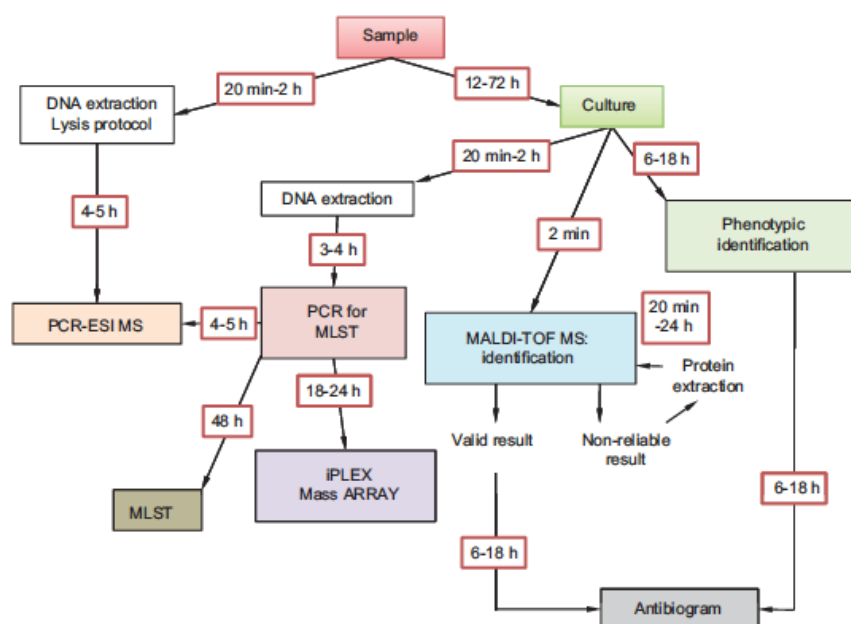


Figure 2 Typical workflow of new and old methods used in a clinical microbiology laboratory. The time to identification, typing and resistance analysis is noted.

MALDI-RE, matrix-assisted laser desorption ionization resequencing; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MLST, multilocus sequence typing; PCR-ESI MS, electrospray ionization mass spectrometry.

Adjustment of the default MALDI-TOF MS database allowed the identification of all members of the *A. baumannii* group as well as other *Acinetobacter* spp. with similar accuracy, as was reported by Espinal et al. [76].

A large number of bacterial cells are required for identification. Usually a whole intact colony is used for analysis, limiting the ability to rapidly identify microorganisms directly from biological fluids where the bacterial count is expected to be relatively low. Research is currently being performed to mitigate some of these requirements.

Other problems could also be noted: 1) the use of MS is challenged by the high costs of the instruments and by the long period of maintenance (up to 2 days) requiring another solution during this period; 2) this technology must be completely adapted to the new constraints of diagnostic laboratories, in particular, the traceability of all the tests; 3) Anderson et al. has demonstrated the effects of some selective solid-medium type on the rate of identification of bacterial isolates by MS [87]. For example, *Staphylococcus* spp. from colistin-nalidixic acid agar medium exhibit low identification rates whereas the same bacteria from blood medium were perfectly identified. In addition to that, it has also been found that protein extraction enhances identification rates and is recommended for colonies grown on different media. However, this extraction increases the time of the experiment; 4) there is also an important need to obtain isolated bacterial colonies to avoid the growth of microorganisms from potentially contaminated material because the technique's ability to resolve mixtures is lacking; and 5) the uncultured bacteria detected by this technology are not mandatory pathogens and must be evaluated with the clinical signs.

It is probable that all these minor inconveniences could be corrected in the future developments. However, one of the most important objectives support improved antibiotic prescription. In fact, this is one of the missing features of this technology and bacterial cultures are still required for antimicrobial susceptibility testing. Therefore, accurate measures and identification of resistance factors could represent the future evolution of this technology.

New developments of the MS

A great number of developments have been made recently to improve the detection limit based on genus and species identification. The MS is now used not only to detect endogenous peptide/proteins to identify bacteria but in the new evolutions, MS enables the improvement of

immunological/PCR detection methods. Moreover, the protein biomarkers that are measured in MS of microorganisms are highly expressed proteins responsible for housekeeping functions, such as ribosomal, chaperone, and transcription/translation factor proteins [88–92]. Based on this detection, new markers have been found and specific databases are being developed for the identification of specific resistance or virulence factors with MALDI-TOF MS technology.

Detection of resistance

Recently some resistance markers to one or more antimicrobial agents have been detected by MALDI-TOF MS. Reports suggest that MS has the ability to differentiate methicillin-susceptible *S. aureus* from methicillin-resistant *S. aureus* (MRSA) strains [93, 94], and also detect carbapenem resistance activity based on the detection of degradation of β -lactam antimicrobials [20, 21, 95]. Basically, the MS follows the enzymatic hydrolysis of the antimicrobial agent. The sensitivity and specificity of this approach is high (97% and 98%, respectively) [21] and the results are available in <3 h [95]. However, this approach must be validated in routine laboratories to consider replacing the conventional techniques (such as cefoxitin disks, or PCR to detect the *mecA* gene). We can speculate that the time frame of MS can be further shortened as suggested by Hooff et al. [20].

Even if the detection of subtle protein alterations will probably be difficult to assess by MALDI-TOF MS, recently, some reports have demonstrated it is possible such as the detection of *rpoB* mutations in *Brucella* sp. [22]. There have also been reports of the detection of bacterial enzymes targeting antibiotics, such as β -lactamases or carbapenemases in *E. coli* and *A. baumannii* [21, 23–25, 95], the CfiA carbapenemase in *Bacteroides fragilis* [26]. Other resistance mechanisms recently reported to be detected by this technology including porin defects and expression of efflux pumps [27].

The ability to use MS technology to rapidly detect the resistance mechanisms produced by a bacterial pathogen will be a key element that will revolutionize clinical microbiology.

Detection of virulence factors

Differences in virulence profiles for bacterial isolates can be based on the selective determination of the presence or absence of *m/z* peaks in the MALDI-TOF MS instrument.

This approach allows not only the identification of the bacterial species, but also to show the presence of some key surface-associated molecules or some well-known virulence factors involving a rapid management of the infection. In this way, the MALDI-TOF MS technology could detect a m/z peak specific to Pantone-Valentine leukocidin-producing *S. aureus* strains, a well-known virulence factor in the development of acute severe *S. aureus* infection [28]. However, a recent publication calls into question the previous work. Szabados et al. claim that protein peaks of 4448 and 5302 Da are not associated with the presence of Pantone-Valentine leukocidin [29].

Some efforts have been made to find biomarkers to differentiate between infectious and non-infectious causes of systemic inflammatory response syndrome (SIRS). Discriminatory peaks have been detected suggesting a direct link between infectious-related protease activity and a sepsis-specific diagnostic pattern for discrimination of patients with SIRS [30]. An interesting example is represented by the detection of the staphylococcal delta-toxin which was found associated with the acute infection [31].

Detection and identification of quorum sensing signals, immune-modulatory proteins and the binding of host factors including antibodies are targets for future research in this field.

Detection directly from samples

Molecular methods have revolutionized clinical microbiology. Indeed a growing range of rapid diagnostic tests that can be performed at the point-of-care has been implemented such as real-time PCR assays [32]. These tools considered to be expensive and time-consuming have clearly evolved and represented a suitable evolution for routine identification. For example, different tests (e.g., GeneXpert™ system or BD GeneOhm™ StaphSR) have been developed for rapid management of contagious diseases such as *Clostridium difficile*, *Bordetella pertussis* or *Neisseria meningitidis* [33, 34]. These tests also allow MRSA to be detected from different sample types (e.g., blood, skin and soft tissue, nasal swabs) and to prevent unjustified prescriptions (e.g., detection of Enterovirus or *Streptococcus agalactiae* in pregnant women [35, 36]).

In this way, the use of MALDI-TOF MS for microorganisms' identification in clinical samples has become essential in the future development of this technology. Some reports have been published recently to detect microorganisms directly from blood or urine samples [37–47]. Methods (combining centrifugation steps, the use of serum separator tubes or ammonium chloride lysis)

for the processing of positive blood culture samples have been proposed to increase the sensibility of the technique but especially in the case of direct testing of other materials (e.g., urine specimens), consensus still has not been reached [37–47]. The analytical sensitivity in blood culture varied between 66% and 76% with a major precision in the identification of Gram-negative bacteria (around 90%) compared to Gram-positive bacteria (<50%). It is of note that current MALDI-TOF MS data software analysis is not able to reliably identify all microorganisms present in mixed cultures. Direct identification of pathogens in urine samples has also been evaluated. The results are not yet satisfactory, the most promising result was obtained for urine containing more than 100,000 CFU/mL [44] and other developments still seem necessary. Different protocols have been used (e.g., concentration step, membrane filtration and magnetic separation) to improve the sensitivity of MS [43, 48]. The use of automation of urine analysis (e.g., urines flow cytometry) in the laboratory in order to eliminate negative samples might render downstream use of MALDI-TOF MS more efficient.

All these evolutions are attractive for the future. Vlek et al. have demonstrated that the direct performance of MALDI-TOF MS on positive blood culture broths reduced the time until species identification by 28.8 h and was associated with an increased proportion of patients receiving an adequate antibiotic treatment within 24 h [49].

PCR/ESI-MS (Table 1)

Other MS solutions have been recently developed to increase the interest of MS technology. The PLEX-ID system (Abbott™) is a nearly fully automated system that associates broad-spectrum PCR (targeting ribosomal and housekeeping protein genes) with electrospray ionization-MS (ESI-MS) (Figure 1) [10]. It delivers broad microbial screening to semiquantitatively identify all organisms present in a sample. It is capable of running multiple human identification targets such as mitochondrial DNA, short-tandem repeat (STR) or single-nucleotide polymorphism (SNP). The principle is to measure the m/z of amplicons, generated by multiplex PCRs that target several loci within bacterial or fungal genomes. The method targets both conserved and species-specific genetic regions to identify microbes based on amplicon base compositions relative to a known database of microorganisms. To date this tool has demonstrated its ability to directly detect and identify bacteria [50] and associated antibiotic resistance genes, such as drug-resistance *M. tuberculosis*, carbapenemase-producing *A. baumannii* or *K. pneumoniae* and

quinolone resistance in *A. baumannii* from isolates [51–55] (Figure 2). Indeed specific primers may be added to the assay to screen the *mecA* gene for methicillin resistance, the *vanA* and *vanB* genes for vancomycin resistance in enterococci, and the *bla_{KPC}* gene for resistance to carbapenems [56–58]. Most recently, the method produced highly accurate results when used to identify bacterial and yeast pathogens directly from the clinical specimen (blood culture) [59], in particular, to detect *Erlichia* isolates [60].

It is important to highlight the technology also offers extended utility for epidemiological surveillance and infection control [51, 52, 54]. It provides quick results (<6 h) and can identify mixtures of up to three to four microorganisms but requires the batching of six samples at a time [10].

In contrast, the first and most limiting step of this technique is DNA extraction from clinical samples. This induces an increase of the costs (2 to 3 times compared to MALDI-TOF) due to the cost of consumables, software package and the need to use DNA extraction reagents (buffers, enzymes, and primers) for PCR.

Although this technology needs to be validated more extensively, there are some recent publications on the detection of *Aspergillus terreus* from bronchio-alveolar lavage [61] and a panel of respiratory viruses from nasopharyngeal aspiration that represents an important target for the future in clinical microbiology [62].

iPLEX MassARRAY® system (Table 1)

The MassARRAY iPLEX single-nucleotide polymorphism (SNP) typing platform uses the MS technology coupled with single-base extension PCR to analyze amplicons of PCR for rapid and accurate molecular identification of microorganisms [63]. This system is commercialized by Sequenom™ (San Diego, CA, USA).

The assay consists of an initial locus-specific PCR reaction, followed by single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using MALDI-TOF MS, the different mass of the extended primer identifies the SNP allele. The starting point of the protocol is the amplification of a target region of interest. T7- and SP6- promoter tagged primers are used to amplify the template. After treatment, *in vitro* transcription provides RNA transcripts which are base-specifically cleaved. The resulting RNA cleavage products are analyzed by MALDI-TOF MS.

The spectra are compared with the simulated spectra of the reference sequences as published for MLST. Due to

the distinct mass of each nucleotide base, the results are as good as those of conventional dideoxy sequencing [64].

This technology has two main applications in clinical microbiology (Figure 2): the comparative sequence analysis, and the SNP genotyping. These two approaches provide a powerful tool in phylogenetic investigation, epidemiology (molecular typing) and surveillance of crossed transmission of bacteria [63, 65, 84]. An interesting example using the MassARRAY technology is the study performed by Syrmis et al. [63] related with the genotyping of MRSA.

MassARRAY iPLEX is more efficient than a sequencing method; however, the analysis by MALDI-TOF MS is much faster than the analysis by capillary electrophoresis, requiring a few seconds for the former one and up to several minutes for the latter one [85]. The iPLEX assay is suitable for high-throughput analysis, as either 96 or 384 samples can be analyzed on the same chip. The major drawback of this technology lies in the requirement for specific equipment and the cost of this equipment.

Other technologies

Other systems for future applications could be developed. One solution includes the surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS system (Table 1). This technology is a specific MALDI-TOF application that combines a chip-based chromatographic enrichment of proteins with TOF-MS [66]. The combination of SELDI (to generate protein profiles and identify significant peaks from large sample sets) and MALDI (to obtain sequence identity of significant peaks) can be extremely powerful for the rapid identification and validation of biomarkers. The SELDI technology incorporates sample prefractionation and binding to the active surface of a 'ProteinChip' array providing more information about the protein of interest than just size, with inferences based on the surface chemistry of the 'chip' (e.g., hydrophobic, reverse-phase, cation-exchange). This design feature of SELDI markedly decreases the complexity of protein-rich fluids such as serum and permits quantitative comparisons of peak intensities between samples using large sample sets [67, 96]. SELDI platforms are specifically designed for the rapid high-throughput comparative analysis of multiple biological samples, increasing the chance of finding proteins with consistently altered expression during disease development, progression or following treatment [96]. The technology allows assessment of the performance of individual biomarkers and to evaluate combinations of biomarkers with potential diagnostic.

The two main limitations of SELDI are its relative imprecision in its assignment of molecular mass to any given peak [67] and its high cost. Very few studies are available concerning bacterial detection increasing the detection limit to the subspecies [66, 97].

A second solution is the liquid chromatography coupled to electrospray ionization triple quadrupole (LC-ESI-QqQ) MS, LC coupled to ESI-Q-TOF (LC-ESI-Q-TOF MS) or MALDI triple quadrupole coupled to MALDI-TOF (Table 1). The LC-ESI-QqQ in selected or multiple reactions way has been used for routine detection of small molecules including metabolites and drugs [31]. More recently, the LC-ESI-QqQ has been suggested as a replacement for classical ELISAs for the quantitation of proteins in complex matrices [98]. The MALDI triple quadrupole measures enzyme-mediated, time-dependent hydrolysis of the β -lactam ring structure of penicillin G and ampicillin and inhibition of hydrolysis by clavulanic acid for clavulanic acid susceptible β -lactamases. This assay represents the basis for future investigations of β -lactamase activity in various bacterial strains [20]. These MS technologies have already been used in research settings extensively and it will be just a brief time before this technology can be introduced in the routine clinical microbiology laboratories. Noteworthy, the most popular routine diagnostic procedure to date was developed two decades ago to facilitate the analysis of solid next to the customary volatile compounds [99]. The technology must be used on liquid and could detect proteins especially peptides. It is completely adapted to quantification of proteins, but, until now, no development has been made in clinical microbiology.

The future: fad or real revolution?

As pointed out in the present review and in previously published papers, MS has clearly revolutionized bacteriological diagnostics. Indeed, currently the delay

between the collection of the specimen and the result of the bacterial culture is a great hindrance to the clinician (Figure 2) [10]. For better use of antibiotics and control of the antimicrobial resistance, a rapid identification of the involved pathogens is of the greatest importance for effective patient management. Indeed, it can reduce the empirical use of broad-spectrum antibiotic therapy to a more narrow specific treatment. However, even if it is promising, MS technology still has limitations which are being overcome. When they are finally resolved, we can definitely speak of a 'revolution'. MS will become a tool for the detection of microbial subtyping, antimicrobial susceptibility testing and virulence factors directly in the samples to guide the clinician in his choice of treatment. Indeed, in parallel, molecular biology seems to be more efficient especially at the point-of-care organization and the syndrome panel solutions.

In conclusion, MS is the future of microbiology: helping clinicians with accurate identification of microorganisms will contribute to timely decision-making for most infectious diseases, resulting in the optimal use of antibiotics, the decrease of multidrug resistant bacteria, the reduction of length and costs of hospitalization.

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Discussion

Staphylococcus aureus est une bactérie pathogène responsable d'un grand nombre d'affections sévères une fois la barrière cutanéomuqueuse passée. Cet agent pathogène est régulièrement isolé dans les plaies du pied chez les diabétiques en particulier dans les pays industrialisés. Antérieurement à mon travail de Thèse, l'équipe INSERM U1047 avait décrit l'existence de souches de *S. aureus* présentant des profils de virulence particulier et détectées au niveau des plaies de pied dans cette population diabétique. Ces souches étaient isolées de plaies non infectées/colonisées et présentaient un très faible potentiel de virulence (Sotto *et al.*, 2007 ; Sotto *et al.*, 2008).

Au cours de notre étude, nous avons pu confirmer l'existence de différents marqueurs génétiques permettant la distinction entre souches de *S. aureus* colonisantes et souches infectantes :

- *cap8*, *sea*, *sei*, et *hlgv* : étaient des gènes marqueurs d'infection des plaies ;
- *edinA*, *edinB* : étaient des gènes associées significativement à des plaies infectées sévères avec un mauvais pronostic d'évolution ;
- *lukDE* : était un gène marqueur de colonisation des plaies ;
- les complexes clonaux CC5/CC8 étaient significativement associés à des souches de *S. aureus* isolées de colonisation des plaies du pied ;
- le complexe clonal ST398 était significativement associé à des souches de *S. aureus* responsables d'ostéites du pied.

A partir de ces travaux et de ces constatations, nous avons pu identifier par optical maps et séquençage de génome complet la présence d'un élément génétique associé aux souches colonisantes présentant un faible potentiel de virulence. Cette insertion génétique correspondait à un phage présentant une très forte homologie avec le phage ROSA (donc le rôle reste inconnu). Ce phage était inséré dans un hotspot Φ NM2, connu pour intégrer assez facilement des phages. Nous avons ensuite confirmé le lien entre ce phage inséré et la faible virulence dans des souches de *S. aureus* isolées de plaies du pied chez des diabétiques.

Le rôle joué par les phages dans l'apport de déterminants de virulence n'est pas un fait nouveau. Ainsi la toxine du cholera est codée par un prophage de la famille f1/M13 (Waldor, 1996), alors que la shiga-toxine et la toxine diphtérique sont codées par un phage lambdoïde lysogénique. Egalement, *Neisseria meningitidis* porte un phage (filamentous phage family) responsable de son potentiel de virulence. Chez *S. aureus*, la présence de phage a été démontrée comme jouant un rôle aussi bien dans la

virulence (par exemple le phage associé à la leucocidine de Panton-Valentine), que dans la résistance aux antibiotiques (cassette *SCCmec*) (Feng, 2008). Dans notre travail de Thèse, nous avons montré qu'un phage inséré dans le génome de *S. aureus* diminuait fortement le potentiel de virulence de la souche en bloquant la machinerie d'acquisition du fer. Les difficultés pour expulser ce prophage, les importants réarrangements génétiques observés dans le phage ROSA-like par rapport à la séquence du prophage ROSA et la basse fréquence d'excision du prophage ont suggéré une très grande stabilité de cette séquence dans le génome et semble indiquer que ce phage ne pourrait pas être transféré à d'autres staphylocoques. Aussi, comme pour les facteurs de virulence bactérienne, l'expression des gènes de cette insertion augmente en milieu carencé en Fer.

Une des voies utilisées par le corps humain pour diminuer le risque d'infection est de réduire et de limiter la disponibilité de nutriments essentiels aux bactéries dans un processus nommé « immunité nutritionnelle » (Weinberg, 1974). Le fer est un élément essentiel aux bactéries pathogènes telles que *S. aureus*. Il est utilisé comme co-facteur pour des enzymes dans différents processus cellulaires tels que la synthèse de l'ADN, la respiration et d'autres voies essentielles (Corbin et al, 2008). Durant le processus infectieux et une fois passer la barrière cutanéomuqueuse, la bactérie se retrouve dans un état de stress puisque plus de 90% du fer est séquestré à l'intérieur des cellules (Hammer ND, Skaar EP 2011). Dans ces conditions, *S. aureus* se doit de déclencher l'expression de ses systèmes d'acquisition du fer. Le contrôle de l'expression des systèmes d'acquisition du fer est négativement régulé en condition normale par un régulateur, Fur. *S. aureus* possède 2 voies d'acquisition du fer. La première voie est la voie Isd qui permet d'absorber le Fer sous forme de hème. Différentes études ont montré que cette voie d'assimilation du Fer était essentielle lors du processus infectieux notamment grâce à la protéine IsdB (Grigg JC et al, 2010). La deuxième voie d'assimilation du fer permet d'absorber le fer libre dans le milieu ou complexé à la transferrine notamment grâce à l'activité chélatrice des siderophores (Hammer ND, Skaar EP, 2011). Fur est un régulateur transcriptionnel qui empêche la transcription des gènes impliqués dans l'assimilation du fer en se fixant sur une séquence consensus nommée Fur box. Cette répression est active lorsque la bactérie est dans un milieu enrichi en fer (Beasley FC and Heincichs DE, 2010 ; Mazmanian SK et al, 2003). Dans un milieu pauvre en fer, par contre, la répression par Fur est levée et l'expression des gènes impliqués dans l'assimilation du fer est déclenchée. Au cours de cette thèse, nous avons montré que la présence du phage ROSA-like dans les souches colonisantes affectait la capacité de la bactérie à assimiler le fer. Cette insertion génétique dérégulait tous les gènes régulés par Fur. Ainsi, la souche colonisante portant l'insertion génétique ROSA-like était affectée dans sa capacité à croître dans un milieu sans fer par un blocage de la levée de restriction de Fur alors que les souches infectantes (qui ne contiennent pas l'insertion génétique ROSA-like) étaient capables de croître dans ce même milieu carencé en fer par la levée de la régulation de Fur et donc l'expression des gènes du locus *isd* ainsi que par sécrétion des sidérophores (Figure P. 121). Nous avons confirmé cette hypothèse en obtenant une souche colonisante ayant perdu son insertion génétique ROSA-like : NSA1385(P-). Cette souche est capable de croître dans un milieu carencé en fer par restauration de la levée de régulation par

Fur et l'expression des gènes du locus *isd* et la sécrétion des sidérophores. Ces données suggéraient que l'insertion du phage ROSA-like affectait le régulateur majeur Fur.

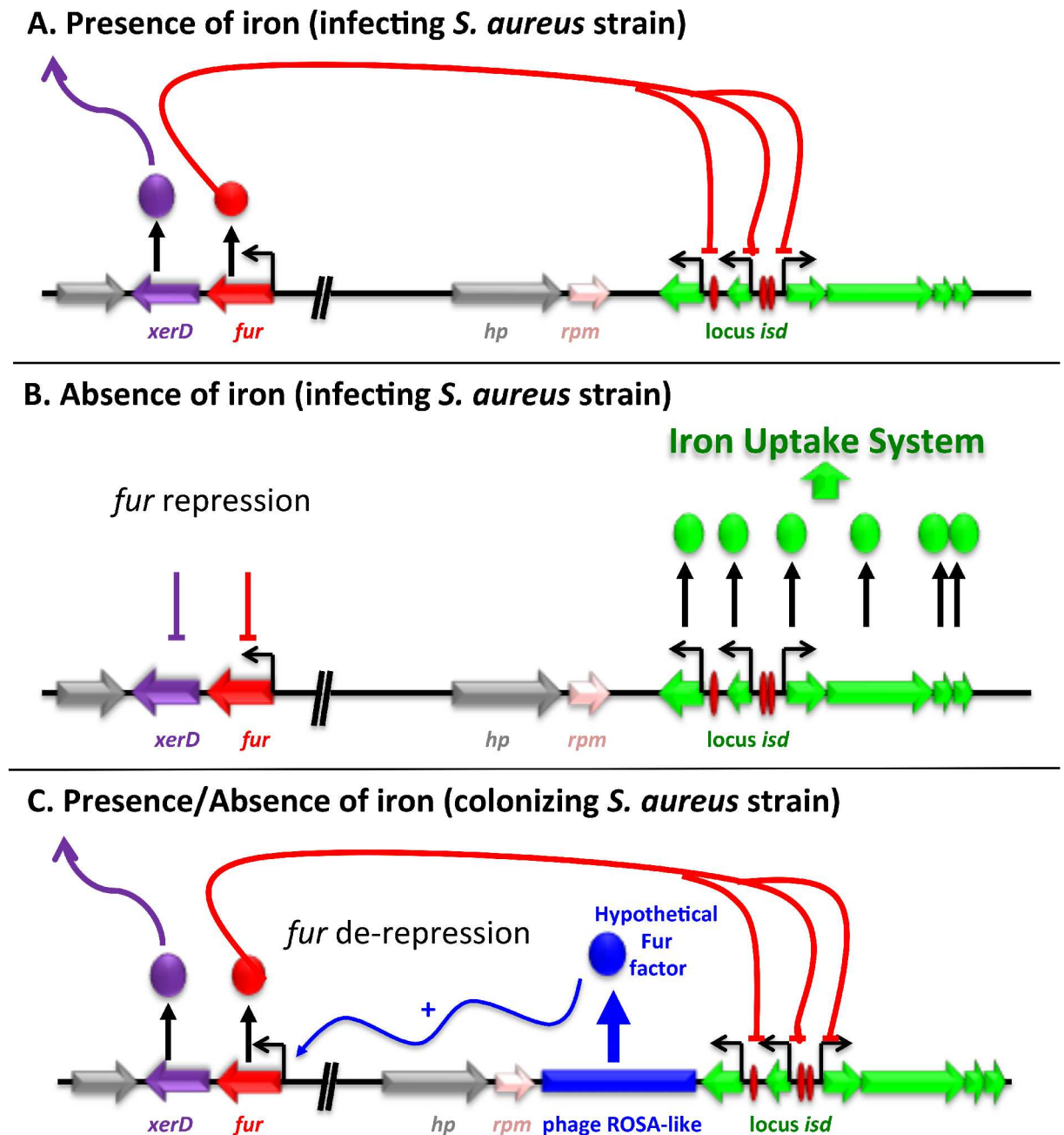


Figure 12. Représentation schématique de la régulation du locus *isd* par la protéine Fur en présence (A,C) et en absence (B, C) de fer chez les souches de *Staphylococcus aureus* infectantes et colonisantes.

A. Condition avec fer pour la souche infectante de *S. aureus*. Le gène *fur* est exprimé induisant la répression du locus *isd* par fixation sur la Fur-box.

B. Condition limitée en fer pour la souche infectante de *S. aureus*. L'absence de fer induit une répression du gène *fur*, les gènes *isd* sont exprimés déclenchant l'activation des systèmes de capture du fer par la bactérie.

C. Condition en présence/absence de fer pour la souche colonisante de *S. aureus*. Une protéine inconnue codée par un/des gène(s) présent(s) dans le phage ROSA-like est constitutivement exprimée induisant la dérégulation du gène *fur*. Les gènes du locus *isd* sont réprimés ne permettant plus l'assimilation du fer et diminuant la virulence.

Plusieurs études ont déjà démontré le rôle des bactériophages dans l'acquisition de facteurs de virulence en intégrant ces phages de manière stable dans le génome (Boyd EF, 2012 ; Brussow H et al, 2004). Récemment, d'autres études sont venues montrer que certains bactériophages pouvaient affecter la virulence des bactéries en empêchant la production de toxines, comme dans le cas de *Clostridium difficile* où le régulateur transcriptionnel RepR codé par le phage ϕ CD119 réprime directement la transcription de gènes codant pour des toxines (TedA et TedB). Chez *S. aureus*, la protéine gp67 codée par le bactériophage G1 perturbe la transcription d'un large nombre de gènes induisant une baisse de la virulence en perturbant la croissance de *S. aureus* (Osmundson J et al, 2012).

L'importance de la capacité à assimiler le fer chez l'hôte a déjà été montré chez plusieurs souches pathogènes telles que *Mycobacterium tuberculosis*, *Helicobacter pylori* et *Neisseria meningitidis* (Reddy PV et al, 2013 ; Boyanova L 2011 ; Livorsi DJ et al, 2011). Chez *S. aureus*, l'importance de la machinerie d'assimilation du fer et l'importance du rôle régulateur de Fur ont déjà été montrées en dérégulant la transcription de Fur, ce qui amène une baisse de la virulence. Dans un premier temps, nos résultats ont montré que l'expression continue de Fur était responsable de la faible virulence de la souche colonisante (NSA1385) en perturbant la machinerie d'assimilation du Fer contrairement aux souches infectantes (NSA739, Newman). Cette observation a été confirmée en testant la virulence de la souche NSA1385(P-) ayant perdu l'insertion ROSA-like. Nous avons alors observé une restauration du potentiel de virulence comparable aux souches infectantes (NSA739, Newman). Dans un second temps, nous avons tenté de restaurer la virulence de la souche NSA1385 en construisant un mutant Δfur . Le but de cette expérience était de restaurer la virulence de la souche colonisante en levant la répression des gènes impliqués dans l'assimilation du Fer. Le résultat obtenu a montré que, malgré l'absence du régulateur Fur, les gènes régulés par ce dernier étaient toujours réprimés chez la souche NSA1385 Δfur , suggérant que la répression des gènes de la machinerie d'assimilation du Fer était réprimée par un facteur présent dans le phage ROSA-like. Ces expériences nous ont amenés à conclure que la dérégulation de Fur était indépendante et concomitante à la dérégulation des gènes impliqués dans l'assimilation du Fer. Ces résultats peuvent être expliqués par l'expression d'un facteur de transcription homologue à Fur ou l'expression d'un potentiel miRNA codé par l'insertion ROSA-like. L'analyse in silico des 73 Kb de la séquence ROSA-like n'ont pas permis d'identifier un potentiel facteur pouvant expliquer la dérégulation de *fur* et la dérégulation des gènes régulés par Fur. D'autres approches, tels que le RNA sequencing ou la construction d'une banque par mutagenèse aléatoire, seront nécessaires pour pouvoir identifier le facteur responsable de la dérégulation des gènes impliqués dans la régulation du fer.

Le potentiel de virulence de *S. aureus* est aussi lié à la capacité de coloniser les tissus de l'hôte et d'établir une infection chronique (Lowy et al, 1998). Dans ce but, *S. aureus* possède d'autres facteurs de virulence comme la capacité à former un biofilm. Un large nombre de protéines de surface produites par *S. aureus* et nommées adhesines de classe MSCRAMMs « Microbial Surface Components Recognizing Adhesive Matrix Molecules » (Foster and Hook, 1998 ; Chavakis et al, 2007) facilite l'établissement d'un biofilm. Ainsi, nombre d'affections telles que les endocardites ou les ostéomyélites sont causées par la capacité de *S. aureus* à former un biofilm. Le biofilm permet aussi une augmentation

de la résistance des souches aux antibiotiques. Le processus d'adhésion et la formation du biofilm sont induits dans les premières étapes de l'infection et il a déjà été démontré que Fur induit la formation du biofilm dans un milieu carencé en fer (Johnson M et *al*, 2005). Nous avons montré que la souche colonisante (NSA1385) avait la capacité à former du biofilm plus rapidement que les souches infectantes (NSA739, Newman) et plus spécialement dans un milieu carencé en fer. Ces résultats confirment que chez la souche colonisante, nous avons une expression continue de *fur* qui augmente la rapidité à former un biofilm. Par ailleurs, des tests d'adhésion faits en collaboration avec l'équipe d'Emmanuel Lemichez (INSERM U1065, Nice) ont montré que les souches colonisantes avaient un faible potentiel d'adhésion par rapport aux souches infectantes dans un modèle de kératinocytes KHN (Figure 13).

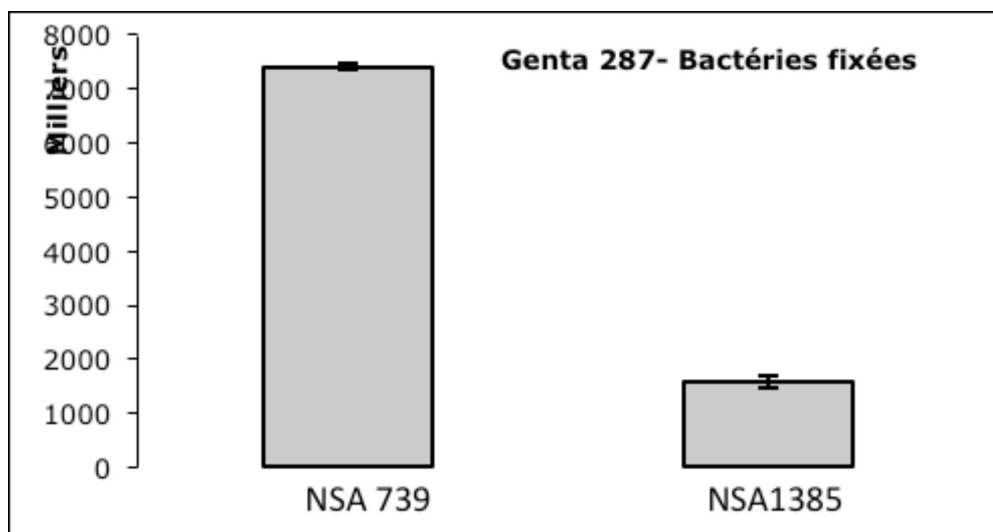


Figure 13. Dénombrement des souches de *S. aureus* adhérentes aux kératinocytes KHN après 2h d'adhésion et lavage à la gentamicine.

Protocole expérimental : Ensemencement des cellules KHN dans 2 plaques 24 puits (8000 cellules/puit) sur feeder de fibroblastes irradiés. Culture sur la nuit des souches de *S. aureus* : 1 colonie/2ml de LB, 37°C et agitation. L'infection était réalisée dans 3 puits de cellules par souche de *S. aureus* avec 10 ml de bactéries (culture de nuit) par puit (mix de 30 ml de bactéries / 1,5 ml de milieu KHN sans antibiotiques, répartition de 500 µl de mix par puit). Les plaques étaient centrifugées 2 minutes à 1000g, à température ambiante. Après 2h d'adhésion à 37°C et 5% CO₂, les cellules étaient lavées 3 fois en PBS puis lysées dans 500 µl de PBS-Triton 0,1%. Une dilution en LB des lysats (au 1/10000) était étalée sur boîte de LB-agar pour quantification des bactéries associées aux cellules. Un comptage des colonies sur boîte était réalisé le lendemain.

Ces résultats expliquent donc pourquoi cette bactérie colonisante peut persister dans l'environnement mais a des difficultés à s'implanter sur la peau ou les muqueuses. Cette bactérie semble avoir sa niche au niveau des plaies chroniques (sa prévalence est de l'ordre de 20%) en participant au biofilm pathologique présent mais sans pouvoir pathogène avéré. Cela lui permet ainsi de coloniser lentement la plaie puis de se regrouper en biofilm. Elle pourrait également faire le lit d'autres pathogènes, leur permettant d'infecter une plaie. Ces éléments devront être évalués ultérieurement.

Notre étude permet de mieux comprendre le commensalisme des souches de *S. aureus*. Elle permet également de proposer une nouvelle approche dans le traitement des plaies du pied chez les diabétiques en évitant l'utilisation des antibiotiques dans certains cas. Elle permet aussi d'ouvrir une voie différente dans la lutte contre les pathogènes notamment par l'utilisation de bactériophage.

Communications – Prix

1- Communications affichées/orales dans un congrès international :

Messad N, Lina G, Prasjnar TK, Renshaw SA, Vandenesch F, Skaar EP, Sotto A, O’Callaghan D, Lavigne JP : A chromosomally integrated bacteriophage in colonizing *Staphylococcus aureus* strain – ISSSI, Lyon (F), Août 2012

Rasigade JP, Dunyach-Remy C, Sapin A, **Messad N**, Trouillet-Assant S, Laurent F, Lavigne JP : From colonization to invasion: phage dynamics modulate intracellular virulence in *Staphylococcus aureus* – 24th ECCMID, Barcelona (Spain), April 2014

2- Communications affichées/orales dans un congrès national :

Lavigne JP, **Messad N**, Vidal-Navarro L, Sotto A ; et le Groupe de travail du PHRC Pied Diabétique : Etude multicentrique sur les souches infectantes/colonisantes de *Staphylococcus aureus* isolées des plaies du pied chez le diabétique – 12èmes Journées Nationales d’Infectiologie, Toulouse, Juin 2011

N. Djahmi, **N. Messad**, JL. Richard, S. Nedjai, A. Moussaoui, D. Mazouz, A. Sotto, JP Lavigne : Epidémiologie moléculaire des souches de *Staphylococcus aureus* isolées des plaies du pied chez le diabétique en Algérie - Paris, Novembre 2012, 32^{ème} Réunion Interdisciplinaire de Chimiothérapie Anti-Infectieuse

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