



Staphylococcus aureus et infection du pied diabétique

Christelle Ngba Essebe

► To cite this version:

Christelle Ngba Essebe. Staphylococcus aureus et infection du pied diabétique. Médecine humaine et pathologie. Université Montpellier, 2016. Français. NNT : 2016MONTT052 . tel-02986601

HAL Id: tel-02986601

<https://theses.hal.science/tel-02986601>

Submitted on 3 Nov 2020

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THÈSE

Pour obtenir le grade de
Docteur

Délivrée par l'Université de Montpellier

Préparée au sein de l'école doctorale 168 :
Sciences Chimiques et Biologiques pour la Santé (CBS2)
Et de l'unité de recherche INSERM 1047

Spécialité : Microbiologie

Présentée par Christelle NGBA ESSEBE

***Staphylococcus aureus* et infection du pied
diabétique**

Soutenue le 30 Novembre 2016 devant le jury composé de

Monsieur le Pr Frédéric LAURENT
Monsieur le Dr Patrice FRANCOIS
Monsieur le Dr Emmanuel LEMICHEZ
Monsieur le Pr Jean-Philippe LAVIGNE

Rapporteur
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DEDICACES

Je dédie cette thèse à ma famille : **mon père Adamou NGBA, ma mère Olive Félicité NGBA, ma sœur Sandrine NGBA** sans laquelle je n'aurais pas été en mesure de finir cette thèse. Les mots ne sont pas assez forts pour vous exprimer ma reconnaissance. Sans votre soutien, votre amour et vos sacrifices je ne serai sûrement pas arrivée jusqu'ici. Je vous remercie pour tout ce que vous avez fait pour moi depuis ma naissance et pour ce que vous continuez de faire tous les jours. Ce travail est pour moi une façon de vous dire MERCI pour tout Je vous aime.

REMERCIEMENTS

Je remercie tout d'abord les membres du jury : **Patrice François, Frédéric Laurent, Emmanuel Lemichez, Eric Ghigo** d'avoir accepté de lire et juger ce travail. Je vous remercie pour le temps que vous y avez consacré.

Aux membres de l'équipe INSERM : **David O'Callaghan, Annette Vergunst, Christine Felix**, Christine Carrio, Albert Sotto et Gisèle Blossier, Philippe Berta, les étudiants Jeremy Ranfaing, Yara Tasrini, Prissana'la folle', Gabriel Bragagnolo, Kevin Reant, Noémie Cadet, Jean-Jacques Tudesq ; Soledad, Nazareth, Nathan, Quentin, mes sœurs masteriennes Clotilde Chatre, Nathalie Pansu, Anaïs Andreo, Amal Mehmoden merci pour ces moments passés ensemble. Chacun de vous a œuvré à l'élaboration de cette thèse par vos conseils, votre aide dans les manipes, les présentations et je tiens à vous dire merci pour cela.

Anne Kériel, je tiens à te remercier pour tous tes conseils durant ces trois années. Ta rigueur scientifique et ta façon d'être m'ont beaucoup appris et je te remercie d'avoir toujours répondu présent lorsque mes encadrants n'étaient pas disponibles.

Alix Pantel, merci du fond du cœur pour la gentillesse dont tu as fait preuve envers moi depuis que je t'ai connu. Malgré tes occupations à l'hôpital tu t'es toujours montré disponible chaque fois que j'en avais besoin. Au-delà de ça je te remercie d'avoir pris le temps de me faire découvrir les magasins exotiques de Nîmes en dépit du froid et du fait que tu aies un nourrisson sur les bras.

Je tiens également à remercier mes amis : **'my love' Lili Zhang, 'my alligator' Karellen Garcias, the 'fakexican' Elia Riquelme, 'the nicest' Joana Feliciano, 'my lovely racist girl' Margarida Gomes, 'mon algérienne préférée' Soumia Brahmi, 'BQ' Pedro Soler**, pour tout votre soutien et votre aide tout au long de ces années. Plus que des amis vous avez été et vous êtes ma famille. Chacune d'entre vous à sa façon m'a aidé humainement et scientifiquement parlant et a contribué à l'élaboration de cette thèse. Vous avez été présentes à chaque fois que j'en avais besoin, vous avez su me donner le sourire, essuyer mes larmes, me faire découvrir de nouvelles saveurs, de nouvelles cultures ...et vous m'avez fait améliorer mon niveau d'anglais. Merci pour tous ces moments de joie passés ensemble. Je n'aurai pas pu rêver avoir mieux comme amis.

Merci aux biologistes et techniciens du laboratoire de microbiologie du CHU de Nîmes pour votre aide technique et vos indications lorsque j'avais des manipes à faire dans votre laboratoire.

A tous les collaborateurs qui ont participé à ce travail : **S Renshaw, S Foster, T Prajsnar**, de l'Université de Sheffield pour votre aide sur la partie mutant ; **Patrice François** de l'hôpital universitaire de Genève, pour m'avoir fait les microarray ; **Orane Visvikis, Emmanuel Lemichez**, Marguerite Fines-Guyon, **Vincent Cattoir, Anne Vergne**, Alain Lecoustumier pour les souches et vos idées sur les expériences avec les bactéries *Helcococcus kunzii*.

A tous ceux qui ont œuvré de près ou de loin à l'élaboration de ce travail je dis merci : Nourredine Messad, François Vandernes, Gérard Lina et tous ceux dont les noms ne sont pas cités.

Je remercie infectiopôle, en particulier **Didier Raoult** de m'avoir donné la possibilité d'effectuer cette thèse en m'offrant un financement. Merci à Micheline Pitaccolo pour son soutien administratif.

A ma famille proche, **Sandrine NGBA, Olive Félicité NGBA, Adamou NGBA**, mes oncles et tantes en particulier **Catherine Elong, James Kuate, Valery Kuate, Jimmy Kuate, Sandra Kuate, Patricia Kuate, Agnès Kuate, Adjara, Patrick Abane, Pius Eya, Felix Lema, Louise Mabom, Jeanne-Flore Kwedi**, Max et Gisela Bourgeois, mes grand-parents Essebe Thérèse et Ebénézer Ndoumbe Mbappe, mes cousins en particulier **Nasser Betchem, Nadège Lema, Cédric Lema**, Léonie Ndoumbe, **Djibril, Habib, Sheila, Anita Djobo**, Patou et Carine PONDY et mes meilleures amies **Christelle ANABA, Juliette Minyono, Claudia Abeng** je vous dis merci pour tout. Vous avez été là dès le début et vous m'avez toujours soutenu chacun à votre façon dans tous les domaines. Si j'ai pu arriver jusque-là c'est grâce à vous et je vous suis infiniment reconnaissante.

Sandrine NGBA tu es ma seule sœur, ma confidente, ma meilleure amie. Depuis ta naissance jusqu'aujourd'hui tu m'as toujours protégé, tu t'es toujours sacrifiée pour moi et tu as toujours fais passer mon bonheur avant même le tien malgré mon mauvais comportement. Je n'oublierai jamais cela et je te dis MERCI encore pour tout.

Mes parents, **Olive Félicité NGBA** et **Adamou NGBA** pour tous vos efforts et vos sacrifices pour mon éducation et mon bien-être je vous dis merci. Malgré la distance vous avez toujours été présents à mes côtés et m'avez toujours mis en priorité dans vos décisions. Par votre façon

d'être vous m'avez appris à être forte et à me battre pendant les moments difficiles. En plus d'être des parents vous êtes aussi des modèles pour moi.

Mes tontons **James** et **Valery Kuate** ainsi que le reste de la famille Kuate je vous remercie de vous être aussi bien occupés de moi. Vous vous êtes toujours impliqués dans mes études et grâce à vous je ne me suis jamais sentie seule en France. Dans les moments de nostalgie, il me suffisait de vous parler ou d'arriver à Paris pour me sentir bien. Patricia je t'ai connu en France et tu m'as tout de suite ouvert les bras, tu as été une mère et une amie pour moi. Merci pour tout ce que tu m'as appris.

Ma tata **Adjara**, je t'ai connu ainsi que tes enfants ici en France et vous m'avez directement accueilli. Merci pour la gentillesse dont vous avez toujours fait preuve à mon égard et pour votre soutien ces durant cette thèse.

Nasser Betchem « Nass ! », mon cousin chéri. Tu as suivi toute l'histoire de cette thèse du début à la fin tu m'as toujours soutenu et aidé. Tu as été là dans les moments difficiles pour moi, tu m'as toujours protégé et nous avons partagé de très bons moments ensemble. Je te remercie pour tout.

Nadège Lema et **Cédric 'Ngba' Lema**, on a partagé des moments forts ensemble que je ne pourrai oublier. Merci pour toutes ces discussions, ces débats, vos conseils, votre aide et votre soutien durant ces années.

Christelle ANABA, ma jumelle du Maroc, jusqu'à présent je ne sais pas comment nous avons fait pour être si proches vu les débuts chaotiques de notre relation. Au Maroc nous avons tissé plus que des liens d'amitié et malgré la distance géographique durant mes années de thèse tu as toujours su me reconforter et répondre présent pour moi quand j'en avais besoin. Merci pour ton soutien et surtout pour le beau présent que tu m'as fait en me faisant l'honneur d'être la marraine de notre beau petit Juan Astyanax.

Juliette Minyono, **Claudia Abeng**, plus que des amies vous êtes mes sœurs de longue date. Nous avons eu comme toutes les sœurs quelques moments de discordes mais vous avez toujours été là pour moi. **Claudia Abeng** « Copine » comme on s'appelle j'aimerais te dire merci parce que tu as été un pilier durant cette thèse. Tu as été là pour écouter mes plaintes incessantes, pour essuyer mes larmes quand ça n'allait pas, pour me faire rigoler grâce à ta folie à toute épreuve. Ma chère **Juliette Minyono** « Bétha » comme j'aime t'appeler, tu es la plus ancienne de mes meilleures amies et malgré la distance géographique depuis le

baccalauréat nos liens n'ont jamais été rompus et tu as été d'un grand réconfort pour moi durant ces dernières années.

A tous mes autres amis en particulier : **Claude Fendzi, Kristian-Michel Mpondo Doualla, Samuel Ngoho, Yann Ngomo, Cedric Ezo'o**, Thierry Essame, Abdouraman Diallo, Daniel Mouthe, James Tombi, Ludovic Anangfack, Hajar Chihab, mes promos du Maroc et tous mes amis éparpillés dans le monde je vous remercie pour votre soutien durant toutes ces années.

Samuel Ngoho « Samychou » comme j'aime t'appeler, on s'est retrouvés en France et depuis tu n'as cessé de m'aider. Je te remercie pour ton écoute, tes conseils, ta disponibilité. Tu es un ami précieux et je suis contente de te compter parmi mes amis.

Yann Ngomo « Yanou », **Kristian-Michel Mpondo Doualla** « KM », malgré la distance vous avez toujours été là pour moi et je vous remercie du fond du cœur pour ça.

Claude Fendzi, je te connais depuis très peu de temps mais tu m'as beaucoup appris. Merci pour tes conseils, ton aide, ton soutien et ta gentillesse.

Catherine Dunyach-Remy, « ma mère du labo » comme j'aimais le dire, sur les papiers ton nom n'apparaît pas mais tu es également mon encadrante. Plus que ça tu as endossé le rôle d'amie et de mère pour moi quand il le fallait. Dès le début tu m'as fasciné par ton intelligence, ta beauté, ton dynamisme, ta gentillesse, ta force ...qualités que l'on voit rarement chez une seule personne. Par la suite tu as fait preuve de beaucoup de patience envers moi et de gentillesse, tu m'as formé sur le plan professionnel et je tiens à te remercier du fond du cœur pour ça. Alors que ma famille était loin de moi après mon opération, tu as été une mère, tu m'as ramené chez moi, tu as fait les courses pour moi, contacté l'infirmière, payé mes médicaments...je ne te dirai jamais assez merci pour ça. Je te remercie pour tout ce que tu m'as appris humainement et techniquement et de m'avoir soutenu surtout psychologiquement tout au long de ces trois années de thèse.

Je tiens à remercier le **Professeur Jean-Philippe LAVIGNE** pour m'avoir donné la possibilité d'effectuer cette thèse. Cher « Professeur », comme j'aime vous appeler, je voudrais à travers ces quelques mots vous remercier de m'avoir encadré tout au long de cette thèse, d'avoir toujours répondu présent quand j'avais des difficultés quelque soit le jour ou l'heure à laquelle je vous écrivais. Je vous remercie pour tout ce que vous m'avez appris et pour vos précieux

conseils. Merci pour votre patience et pour le fait que vous ne me mettiez jamais la pression. Je vous remercie pour votre gentillesse, vous m'avez beaucoup fait rire durant ces années et alors que je méritais souvent d'être grondée vous avez toujours fait passer vos messages même les plus importants de façon comique. Votre dynamisme et votre intelligence font de vous un modèle de travail à suivre pour moi et je suis très reconnaissante de vous avoir eu comme directeur de thèse.

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LISTE DES ABREVIATIONS

Gènes et protéines

Agr : *accessory gene regulator*
CHIPS : *chemotaxis inhibitory protein of Staphylococci*
Coa : *coagulase*
Eap : *extracellular adherence protein*
Emp : *extracellular matrix binding protein*
EDIN : *epidermal cell differentiation inhibitors*
Fnbp : *fibronectin binding protein*
Fur : *ferric uptake receptor*
Hla : hémolysine alpha
Hlb : hémolysine bêta
Hlg : hémolysine gamma
Isd : *Iron-regulated surface determinant*
MSCRAMM : *microbial surface components recognizing adhesive matrix molecule*
Orf : *open reading frame*
PIA : *polysaccharide intercellular adhesin*
PVL : *Panton-Valentine leukocidin*
Sar : *staphylococcal accessory regulatory*
SEs : *staphylococcal enterotoxins*
Spa : protéine A
TSST-1 : *toxic shock syndrome toxin*

Généralités

ARN : acide ribonucléique
ADN : acide désoxyribonucléique
ATP : Adénosine triphosphate
C. elegans : *Caenorhabditis elegans*
DFU : *Diabetic foot ulcers*
H. kunzii : *Helcococcus kunzii*
IPD : infections du pied diabétique
LB : *Luria broth*
LT50 : *lethal time 50*
PCR : *Polymerase chain reaction*
qRT-PCR : *Real-Time Quantitative Reverse Transcription PCR*
UPD : Ulcères du pied diabétique

INTRODUCTION GENERALE

I. Diabète sucré

A. Définition

Le diabète est une maladie caractérisée par une forte teneur en glucose dans le sang due à un défaut de production d'insuline et/ou à un défaut d'action de l'insuline ¹. C'est un problème mondial tant sur le plan médical que social car c'est la troisième cause de mortalité et de morbidité dans le monde ^{2 3}. Selon la fédération internationale du diabète en 2015, une personne meurt du diabète toutes les 6 secondes ⁴.

B. Classification

Il existe plusieurs types de diabète sucré :

- Le **diabète de type 1** : environ 6% des cas de diabètes traités sont des diabètes de type 1. Celui-ci se caractérise par la destruction des cellules β des îlots de Langerhans du pancréas entraînant une carence absolue en insuline. Deux sous-types existent :

(i) le diabète de type 1 auto-immun : il survient généralement chez le sujet jeune bien qu'il puisse apparaître à tous les âges. La destruction des cellules β par un processus auto-immun est due à des anticorps anti-cellules d'îlots, anti-insuline, anti-glutamate décarboxylase (GAD), anti-tyrosine phosphatase IA-2 et IA-2b.

(ii) le diabète de type 1 idiopathique : dans cette forme, la composante héréditaire est importante. Il concerne une minorité de personnes en particulier des sujets d'origine africaine ou asiatique.

- Le **diabète de type 2** représente la majorité des cas de diabètes traités 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** englobe les troubles de la tolérance glucidique, de sévérité variable, débutant ou diagnostiqué pendant la grossesse.

Les **autres diabètes** sont liés à des défauts génétiques de la fonction de la cellule β ou à des défauts génétiques de l'action de l'insuline. Ce sont les diabètes induits par une

pancréatopathie, les diabètes iatrogènes, les diabètes induits au cours d'infections, les formes rares de diabètes liées à un dysfonctionnement du système immunitaire ou encore les diabètes accompagnant divers syndromes génétiques.

C. Critère diagnostique 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 »⁵.

Différents biomarqueurs sont utilisés pour définir le diabète incluant la glycémie à jeun, le test de tolérance au glucose et plus récemment, l'HbA1c^{1,6}.

D. Complications du diabète sucré

Environ 70% des patients diabétiques de type 2 présentent des complications à plus ou moins long terme (Figure 1)⁷. 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 de la perméabilité, du débit et de la pression capillaire, une perte de l'autorégulation hémodynamique avec vasoplégie artériolaire d'amont, une augmentation de la viscosité sanguine et une tendance thrombogène avec notamment une augmentation du facteur de Willebrand synthétisé par les cellules endothéliales. 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.

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 1 : 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) ⁷.

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

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

La **macroangiopathie** désigne l'atteinte des artères de moyen et gros calibre. Elle comprend les atteintes des artères coronaires, des artères à destination 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. Les complications de l'hyperglycémie chronique dans le sang sont nombreuses. Elles incluent des complications vasculaires affectant les yeux, les reins, les nerfs ainsi que l'augmentation des risques des maladies cardiovasculaires ¹.

II. Pied diabétique

A. Définition

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.

Les patients diabétiques sont donc sujets à de nombreuses complications: atteintes vasculaires périphériques, ischémie, neuropathie ⁸⁻¹¹, artériopathie ² dont l'association induit généralement des ulcères du pied diabétique (UPD). Les ulcères du pied sont fréquents chez les patients diabétiques, avec une prévalence qui s'élève à 25% ¹²⁻¹⁴. Quarante à quatre vingts pour cent de ces ulcères finissent par devenir infectés ¹⁵ et la propagation de l'infection aux tissus mous et aux structures osseuses est un facteur causal majeur d'amputation des membres inférieurs ^{12,14-16 17}. 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). Ces infections du pied diabétique (IPD) sont une cause majeure de morbidité et d'hospitalisation ^{8,18} ce qui fait du pied diabétique un problème majeur dans tous les pays tant sur le plan médical que social et économique ².

B. Causes d'ulcération du pied diabétique

La survenue d'une infection est toujours secondaire à une plaie du pied ¹⁹. La fréquence et la sévérité des IPD trouvent leur origine dans :

-l'altération des fonctions des polynucléaires neutrophiles, particulièrement marquée en cas d'hyperglycémie prolongée ¹⁹ ;

- l'anatomie particulière du pied qui favorise la dissémination de l'infection ^{10,19} : augmentation de la pression plantaire, cas d'hyperpression plantaire ^{2,20} (Figure 2) ;
- la neuropathie ⁸⁻¹¹ qui rend la plaie du pied non douloureuse et en réduit les signes d'inflammation, l'ensemble concourant à retarder considérablement le diagnostic ¹⁹ ;
- l'ischémie ^{8,10}, l'artériopathie ² considéré comme le résultat d'une hypoxie nerveuse secondaire à une maladie microvasculaire et d'un effet direct de l'hyperglycémie ^{9,20}.
- les facteurs métaboliques : diminution de l'acuité visuelle, surpoids, présence d'une mycose du pied, protéinurie et rétinopathie ² ;
- la susceptibilité génétique, les mécanismes de défense immunitaire humorale altérés, lésions nerveuses ²¹.

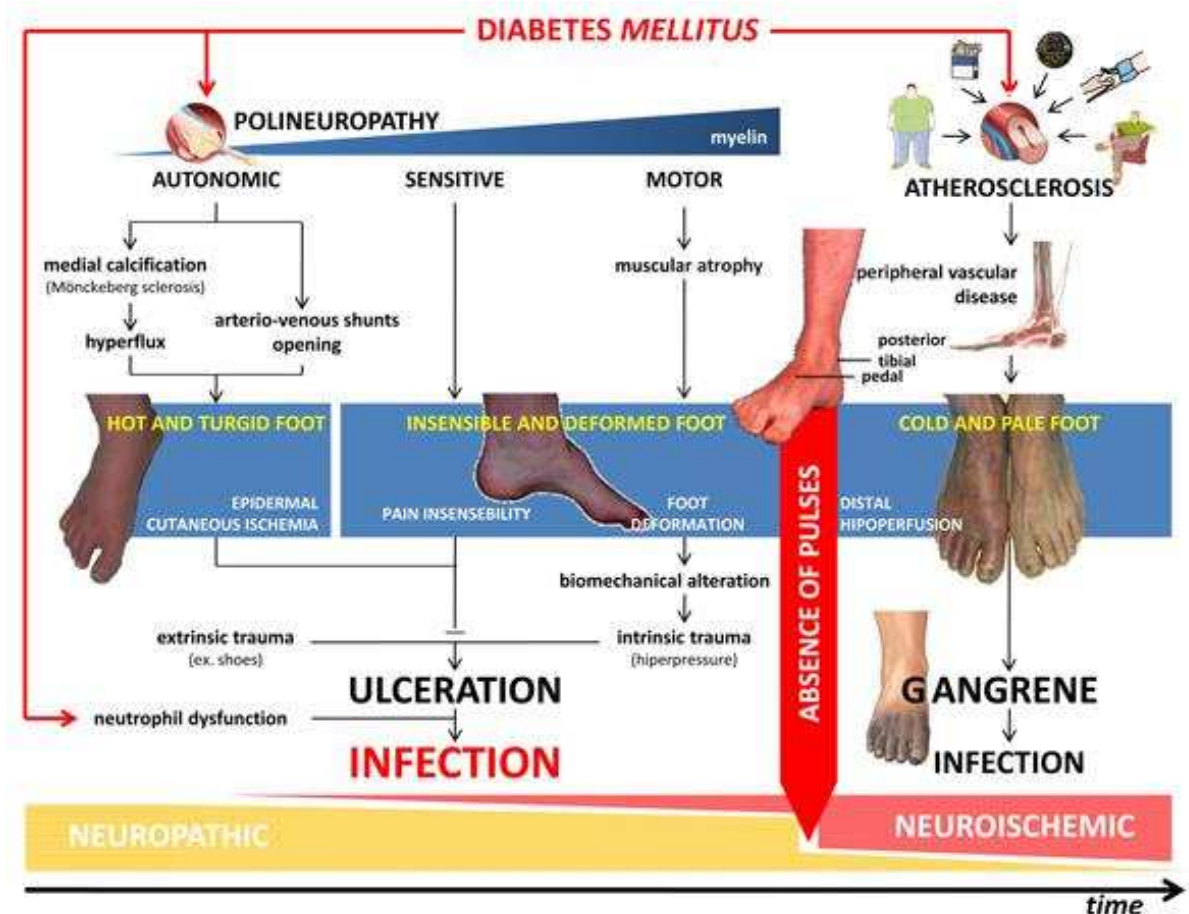


Figure 2 : Différentes causes d'ulcération du pied diabétique ¹⁷.

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. En effet, la plaie est constamment colonisée par des bactéries commensales présentes sur le revêtement cutané du patient et par des bactéries environnementales. 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 ²²:

- 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 ²³. Parmi elles, deux ont été retenues.

La première est celle de l'université du Texas ^{24,25}. Elle comprend quatre grades en fonction de la profondeur et quatre stades dépendant de 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 précisée dans le Consensus International sur le pied diabétique (Tableau 2). L'infection y est caractérisée par la présence d'au moins deux signes d'inflammation (*cf.* ci-dessus). 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 ²⁶ et 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 passer inaperçues chez le patient diabétique (en raison de l'artériopathie et de la neuropathie sous-jacente). D'autres paramètres sont donc indispensables afin d'aider au diagnostic d'infection quand les signes classiques sont absents.

	Grade 0 lésion épithélialiste (%)	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 au moins deux des caractéristiques suivantes : - 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 microbiologiques d'intérêt

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.

Les examens microbiologiques standards ont pour but d'obtenir l'isolement et l'identification de la ou des bactérie(s) responsable(s) de l'infection à partir d'un prélèvement clinique en évitant sa contamination par le microbiote commensal qui colonise la peau.

La Figure 3 a pour but de résumer le choix des prélèvements à effectuer en fonction du type de plaie. Les prélèvements doivent être obligatoirement réalisés sur une plaie débridée. Ce débridement peut-être soit chirurgical (au bloc opératoire par un chirurgien), soit mécanique au moyen d'une curette ou d'un scalpel stériles et nettoyée avec du sérum physiologique.

Concernant l'interprétation des résultats, il est conseillé dans un premier temps de ne pas prendre en considération les bactéries reconnues comme commensales ou comme étant les moins virulentes (staphylocoques à coagulase négative, entérocoques, corynébactéries, *Pseudomonas aeruginosa*)^{22,27}. En cas de doute, les prélèvements devront être répétés et ces bactéries seront prises en compte si elles sont isolées à plusieurs reprises.

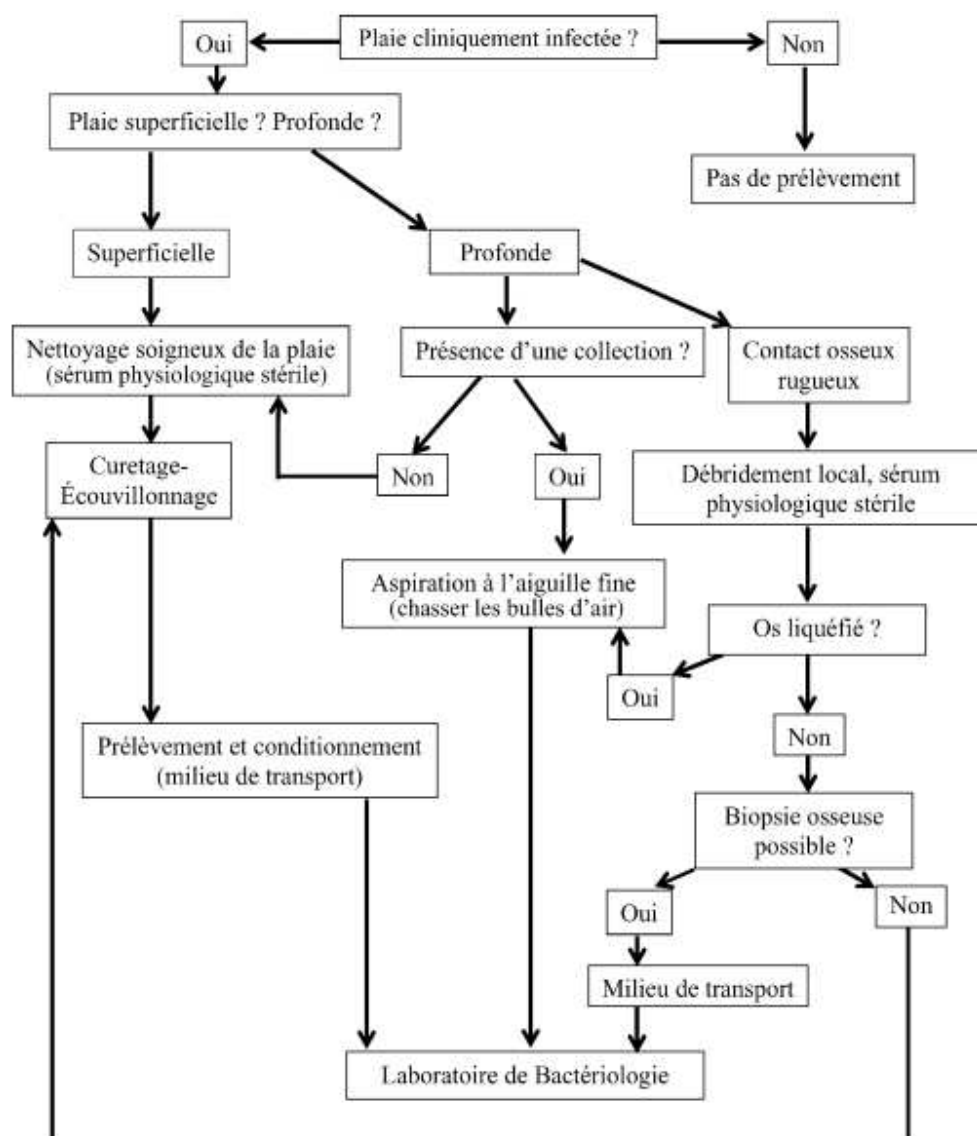


Figure 3: Prélèvements microbiologiques à effectuer en fonction du type de plaie du pied.

D. Ecologie

La lutte contre l'infection du pied diabétique nécessite une connaissance de son microbiote afin de définir la stratégie thérapeutique la mieux adaptée. L'écologie bactérienne des plaies infectées du pied diabétique est la plus souvent polymicrobienne^{28,29}. L'étude OPIDIA a été menée dans 38 centres hospitaliers français. Elle 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 fréquemment isolée (54% des bactéries à Gram positif et 32,5% de l'ensemble des souches isolées). Les streptocoques β -hémolytiques se positionnent à la deuxième place 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 isolées ³⁰. Les bactéries aérobies à Gram négatif isolées appartiennent le plus souvent à la famille des entérobactéries (*Proteus mirabilis*, *Escherichia coli*, *Klebsiella* spp.). Elles sont principalement présentes sur des infections chroniques. *P. aeruginosa* quant à lui, est plus souvent isolé dans des cas d'ostéites que dans les infections des tissus mous ³¹. Cette bactérie est fréquemment isolée en cas d'hospitalisations prolongées. Cependant, son rôle pathogène est discuté. Dans les pays en voie de développement, c'est toutefois la bactérie la plus fréquemment isolée (Asie du Sud Est notamment). Vingt pourcents des patients étaient infectés par des bactéries multirésistantes dont 26% par des souches de *P. aeruginosa* et 25% par des *S. aureus* ³⁰.

Les bactéries anaérobies n'étaient isolées que dans 2% des cas. Ces bactéries 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 la part importante de ces bactéries anaérobies contrairement aux résultats obtenus en culture classique. En effet, ces bactéries sont constamment présentes dans des plaies profondes ³².

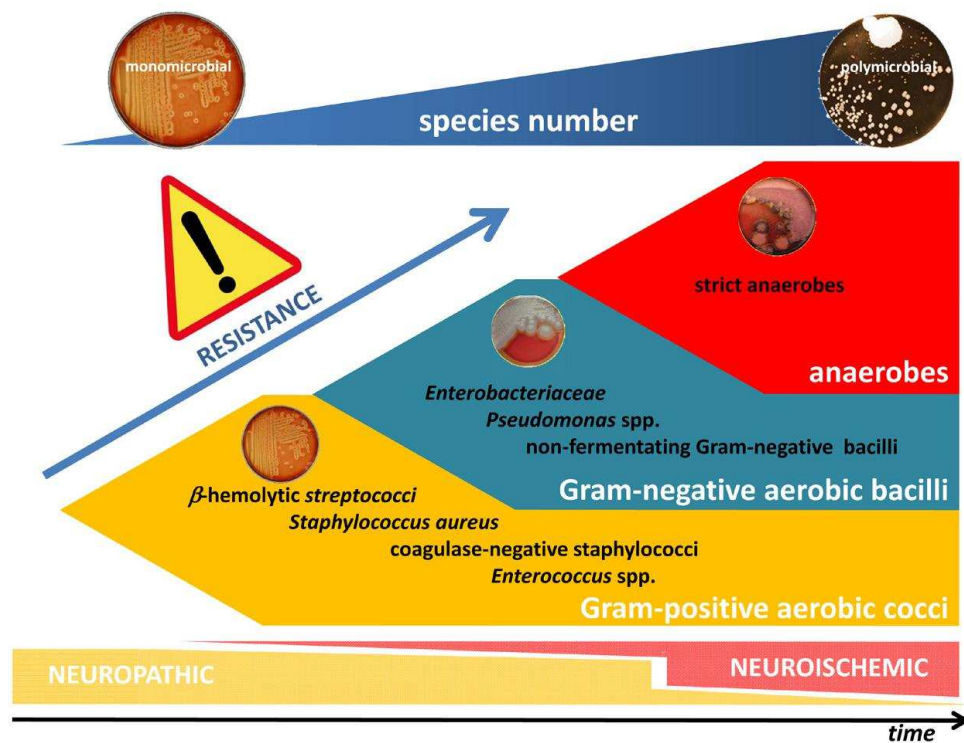


Figure 4 : Ecologie du pied diabétique. ¹⁷

III. *Staphylococcus aureus* dans le pied diabétique

A. Généralités

Comme nous l'avons évoqué précédemment, plusieurs études ont montré que l'écologie du pied diabétique est polymicrobienne : staphylocoques, streptocoques, bacilles à Gram négatif et bactéries anaérobies sont les organismes les plus fréquemment isolés ²⁰. *S. aureus* est la principale bactérie responsable de ces infections ^{12,13,15,33}. Il s'agit d'un cocci à Gram positif, anaérobie facultatif ³⁴. Cet agent pathogène capable de coloniser les narines et les autres muqueuses de l'homme peut être considéré comme un organisme commensal colonisateur. Toutefois, il a la capacité d'infecter presque tous les tissus du corps humain ³⁵ d'où l'importance clinique de cette bactérie. En effet, cette bactérie est responsable d'un large éventail de maladies allant d'infections des tissus mous (furoncles, impétigo) aux infections invasives telles que les pneumonies, les ostéomyélites et l'endocardite ^{21,36}.

Cette bactérie occupe une place centrale en microbiologie. En effet, la prévalence des infections staphylococciques, nosocomiales (associées aux soins), et communautaires dont les infections du pied diabétique, augmente régulièrement. Le traitement de ces infections est parfois difficile à cause de souches multirésistantes aussi bien hospitalières que communautaires bien qu'il y ait une nette diminution des SARM (*S. aureus* résistant à la méticilline), en France ³⁷.

B. Virulence de *Staphylococcus aureus*

Le potentiel pathogène de *S. aureus* est dû à l'expression contrôlée d'un arsenal de facteurs de virulence dans le but de coloniser, d'envahir et de détruire les cellules et tissus ^{36,38}.

1. Les adhésines

Ce sont les composants microbiens de surface reconnaissant les protéines de la matrice de l'hôte (MSCRAMMs) : les protéines de liaison à la fibronectine Fnbp A et Fnbp B, les protéines de liaison au fibrinogène (ClfA, ClfB), la protéine d'adhérence extracellulaire (Eap), la protéine de la matrice extracellulaire (Emp), la coagulase (coa) ³⁹⁻⁴¹, le déterminant de surface régulé par le fer (Isd A), l'acide teichoïque de la paroi (WTA) ⁴², les protéines de liaison au collagène (Cna) ⁴³ (Figure 5).

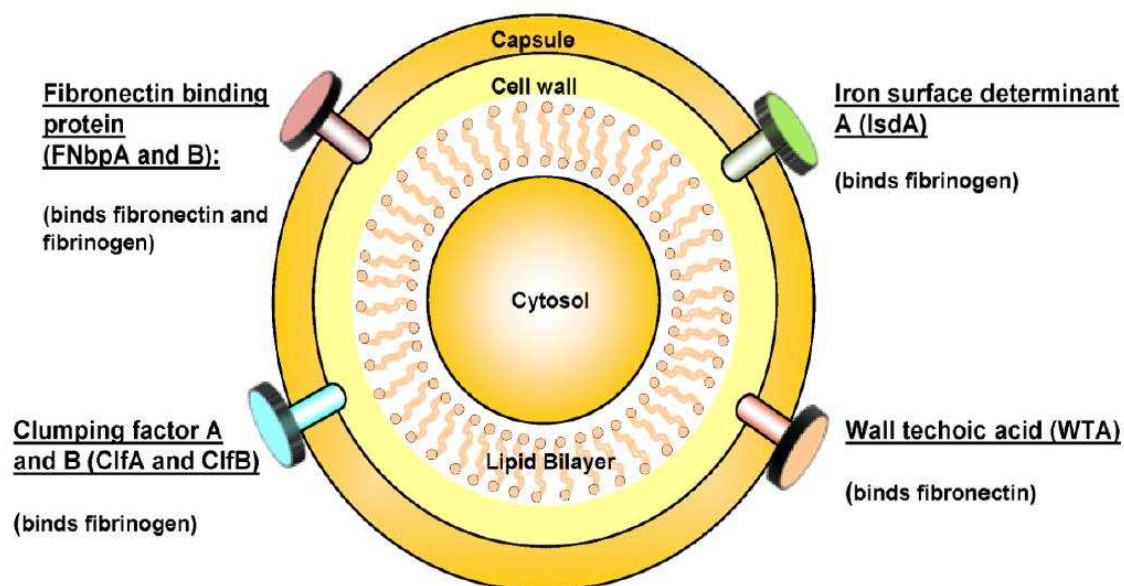


Figure 5: Molécules d'adhésion du *S. aureus* ⁴².

Ils permettent la colonisation par l'adhérence aux composants de surface de la peau tels que le fibrinogène, la fibronectine et les kératinocytes épidermiques.

2. Les toxines et les exoprotéines

Durant une infection, *S. aureus* dispose de plusieurs mécanismes pour tuer les cellules immunitaires de l'hôte, inhiber le recrutement des polynucléaires neutrophiles et réduire la fonction antimicrobienne ⁴². Il s'agit des alpha et bêta toxines (Hla, Hlb), des gamma-toxines (HlgA, HlgB, HlgC) ⁴⁴, des leucocidines incluant la γ -hemolysine, la leucocidine de Panton-Valentine (PVL), la leucocidine E/D ⁴⁵⁻⁴⁷ qui détruisent les cellules et tissus. Les exoenzymes EDINs (EDIN-A, -B and -C) permettent quant à elle la dissémination de la bactérie dans la circulation et les tissus ^{48,49}. D'autre part, les protéases, nucléases, hyaluronidases, lipases ⁵⁰, protéine A (Spa) permettent l'échappement au système immunitaire et la protection contre la clairance des cellules de l'hôte ⁵¹. Il existe également des protéines qui inhibent le recrutement des neutrophiles (CHIPS). Certaines toxines inhibent l'activation des lymphocytes T ; ce sont les superantigènes tels que les entérotoxines staphylococciques A et B (SEA et SEB) et la toxine responsable du syndrome de choc toxique 1 (TSST-1). D'autres toxines telles que les toxines exfoliatives (Ets) facilitent l'invasion de la peau ⁵².

Travail n°1

***Staphylococcus aureus* Toxins and Diabetic Foot Ulcers: Role in Pathogenesis and Interest in Diagnosis**

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Revue : Toxins

Année : 2016

Review

***Staphylococcus aureus* Toxins and Diabetic Foot Ulcers: Role in Pathogenesis and Interest in Diagnosis**

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Academic Editor: Vernon L. Tesh

Received: 14 May 2016; Accepted: 1 July 2016; Published: 7 July 2016

Abstract: Infection of foot ulcers is a common, often severe and costly complication in diabetes. Diabetic foot infections (DFI) are mainly polymicrobial, and *Staphylococcus aureus* is the most frequent pathogen isolated. The numerous virulence factors and toxins produced by *S. aureus* during an infection are well characterized. However, some particular features could be observed in DFI. The aim of this review is to describe the role of *S. aureus* in DFI and the implication of its toxins in the establishment of the infection. Studies on this issue have helped to distinguish two *S. aureus* populations in DFI: toxinogenic *S. aureus* strains (harboring exfoliatin-, EDIN-, PVL- or TSST-encoding genes) and non-toxinogenic strains. Toxinogenic strains are often present in infections with a more severe grade and systemic impact, whereas non-toxinogenic strains seem to remain localized in deep structures and bone involving diabetic foot osteomyelitis. Testing the virulence profile of bacteria seems to be a promising way to predict the behavior of *S. aureus* in the chronic wounds.

Keywords: diabetic foot infection; *Staphylococcus aureus*; toxins

1. Introduction

Foot ulcers are common in diabetic patients. Its prevalence varies between 15% and 25% [1]. Infection of these ulcers is a frequent (40%–80%) complication representing a major cause of mortality and morbidity [2]. It is estimated to be the most common reason of lower-limb amputations [3–5]. The pathophysiology of diabetic foot infection (DFI) is quite complex. The prevalence and severity are a consequence of host-related processes (e.g., immunopathy, neuropathy and arteriopathy) and pathogen-related factors (e.g., virulence, antibiotic-resistance and microbial organization) (Figure 1) [6–8].

DFI pose many problems in clinical practice in terms of both management and diagnosis [9]. Indeed, the presence of impaired leukocyte functions and/or peripheral arterial disease may reduce the local inflammatory response and classical signs or symptoms of local infection [10,11]. Moreover, systemic signs of toxicity (e.g., leukocytosis or fever) may be lacking or appear late, even in severe cases [12–14]. Microbiological diagnosis of these DFI also encounters many limitations. As microorganisms colonize all chronic wounds, the diagnosis of DFI should not be based only on the microbiological analysis of a wound culture, but also on clinical findings [5,9,15]. The Infectious Diseases Society of America (IDSA) and the In-

ternational Working Group on the Diabetic Foot (IWGDF) have developed clinical criteria for classifying the severity of DFI (Table 1) [15,16].

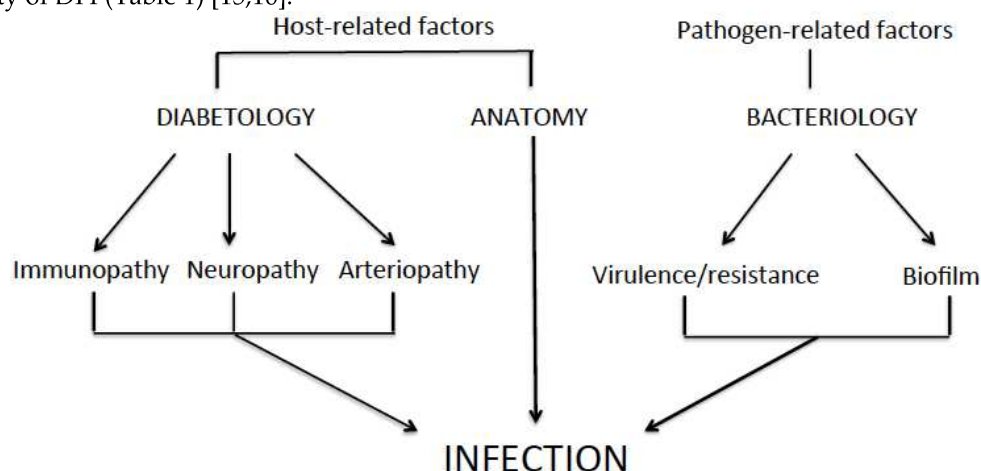


Figure 1. Interactions between metabolic, anatomical and bacteriological factors in diabetic foot infection.

Table 1. International Consensus on the Diabetic Foot classification of foot wound infections [3,15].

Grades	Symptoms
Grade 1	No symptoms, no signs of infection
Grade 2	Lesion only involving the skin (no subcutaneous tissue lesion or systemic disorders) with at least two of the following signs: -local warmth -erythema >0.5–2 cm around the ulcer -local tenderness or pain -local swelling or induration -purulent discharge (thick, opaque to white or sanguineous secretion) Other causes of inflammation of the skin must be eliminated (for example: trauma, gout, acute Charcot foot, fracture, thrombosis, venous stasis)
Grade 3	-Erythema >2 cm and one of the findings described above or -Infection involving structures beneath the skin and subcutaneous tissue, such as deep abscess, lymphangitis, osteomyelitis, septic arthritis or fasciitis There must not be any systemic inflammatory response (see Grade 4)
Grade 4	Regardless of the local infection, in the presence of systemic signs corresponding to at least two of the following characteristics: -temperature >39 °C or <36 °C -pulse >90 bpm -respiratory rate > 20/min -PaCO ₂ <32 mmHg -leukocytes >12,000 or <4000/mm ³ -10% of immature leukocytes

For many decades, culturing wound specimens were the only way to determine the causative pathogen(s) in a DFI. As microorganisms are always present on every skin wound and the DFI are often polymicrobial, the variability of bacterial virulence factors and the level of host resistance must also be

taken into account. In fact, the different organisms isolated from infected wounds do not have a similar pathogenic impact, and evaluation of the intrinsic virulence potential of isolated bacteria to identify their real pathogenicity seems a promising way to best characterize the infection and to distinguish infection from colonization [16].

Several studies have shown that DFI are polymicrobial, and *Staphylococcus aureus* is the pathogen most frequently isolated [17–22]. *S. aureus* is both a commensal bacterium and a human pathogen. Indeed, approximately 30% of the human population is colonized with *S. aureus* [23]. Importantly, this bacterium causes a wide range of clinical infections (e.g., bacteremia, endocarditis, skin and soft tissue, osteoarticular, pulmonary and device-related infections) [24]. The numerous virulence factors and toxins produced by *S. aureus* during infection are well characterized [22]. However, some specific features could be observed in DFI. The aim of this review is to describe the role of *S. aureus* in DFI and the implication of its toxins in the establishment of the infection.

2. DFI and *Staphylococcus aureus*

2.1. Clinical Aspects of DFI

Many DFIs are superficial at presentation. However, bacteria can spread to subcutaneous tissues, including tendons, joints, fascia, muscle and bone. DFIs were classified by their clinical severity, ranging from mild (~35% of cases, depending on site of presentation), through moderate (~30%–60%), to severe (~5%–25%) [25]. The IWGDF and the IDSA have proposed simple clinical criteria for classifying the infection of diabetic foot ulcer (DFU) based on classical signs and symptoms of inflammation (Table 1) [3,15,25–27]. This scheme helps to predict whether hospitalization would be required and the clinical outcome. Moreover, various factors have been suggested as markers of DFI when classical signs are not obvious. These include the identification of friable or discolored granulation tissue, necrosis, fetid odor, non-purulent secretions, delay in healing despite otherwise adequate ulcer management and the discovery of unexplained hyperglycemia [16,25]. Interestingly, neither toxic shock syndrome nor toxinogenic manifestations could be clearly diagnosed in DFI.

2.2. Osteomyelitis

Infection of bone, or osteomyelitis, is found in ~50%–60% of patients hospitalized for a DFI and ~10%–20% of apparently less severe infections presenting in the ambulatory setting [28]. Bone infection results in inflammatory destruction, bone necrosis and new bone formation. It typically involves the fore-foot and develops by contiguous spread from overlying soft tissue, penetration through the cortical bone and into the medullary cavity. The clinical presentation of diabetic foot osteomyelitis (DFOM) can vary with the site, the presence of any associated abscess or soft tissue involvement, the extent of bone infection, the bacterial species and the adequacy of limb perfusion. Osteomyelitis is frequently associated with vascular insufficiency [24,28,29].

Two clinical manifestations are frequently found during DFOM: an ulcer lying over a bony prominence (particularly when it fails to heal despite adequate off-loading) and a ‘sausage toe’. Osteomyelitis can, however, occur in the absence of overlying local signs of inflammation [30].

2.3. Matrix Metalloproteinases and DFI

During DFI, wound healing is hampered by mechanisms including a low growth factor activity, a reduced cellular proliferation, an elevated inflammatory markers and high levels of proteases [31].

The proteases are enzymes that act to control the degradation of extracellular matrix (ECM) [32]. The major group of proteases involved in the wound healing process are the matrix metalloproteinases (MMP) (e.g., MMP-2, MMP-8, MMP-9 and the serine proteases (human neutrophil elastase, HNE)). MMPs are

endopeptidases whose physiological functions are to degrade the different components of the cutaneous tissue (collagen type I, elastin, etc.) and to facilitate keratinocyte migration and re-epithelialization [33,34]. HNE is an enzyme acting on a wide range of proteins in the ECM and on inflammatory mediators [31]. The MMPs' activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs) [35]. The balance between the level of proteases and their inhibitors is essential to allow a physiological healing process [36].

In DFI, under the hypoxic and inflammatory environment, the presence of elevated levels of MMPs could be noted as opposed to decreased levels of TIMPs [36–39]. This elevated protease activity participates in the important destruction of the ulcer ECM. It impairs the release of the different factors regulating the wound healing process (the collagen synthesis is deregulated; the growth factor synthesis and action are stopped) and affects extracellular matrix components, such as fibronectin [40]. All of these elements stall the wound in a chronic inflammatory phase without progressing to healing. However, no clear link between delayed healing and elevated protease activity has been described. This reinforces the need to understand the organization/cooperation between bacteria species that can modulate local inflammation and host MMP production.

In addition to human MMPs, some bacteria produce proteases that have a role in the healing of infected wounds. For example, the zinc-metalloproteinase, elastase, produced by *Pseudomonas aeruginosa*, induces degradation of fibroblast proteins and proteoglycans in chronic wounds and has also been shown to degrade host immune cell mediators. The microbial proteases participate also in the degradation of human ulcer fluid and inhibit fibroblast growth. It has now been suggested that human and bacterial MMPs act synergistically to maintain the lesion in a chronicity status [32]. Many pathogenic bacteria isolated on wounds (including *S. aureus*) are able to produce metalloproteinases.

2.4. Prevalence of *S. aureus* in DFIs

In Occidental countries, Gram-positive aerobic cocci are the main microorganisms responsible for DFI with *S. aureus* the most commonly isolated bacteria, alone or in combination, in superficial or deep infection (Figure 2) [19,20,29,30,41–72]. In warmer countries (particularly in Asia and Africa), Gram-negative bacilli are more prevalent.

Many cases of deep infections and DFOM are polymicrobial. Also in this case, *S. aureus* is the main isolated bacteria, present in 30%–60% of cases [25].

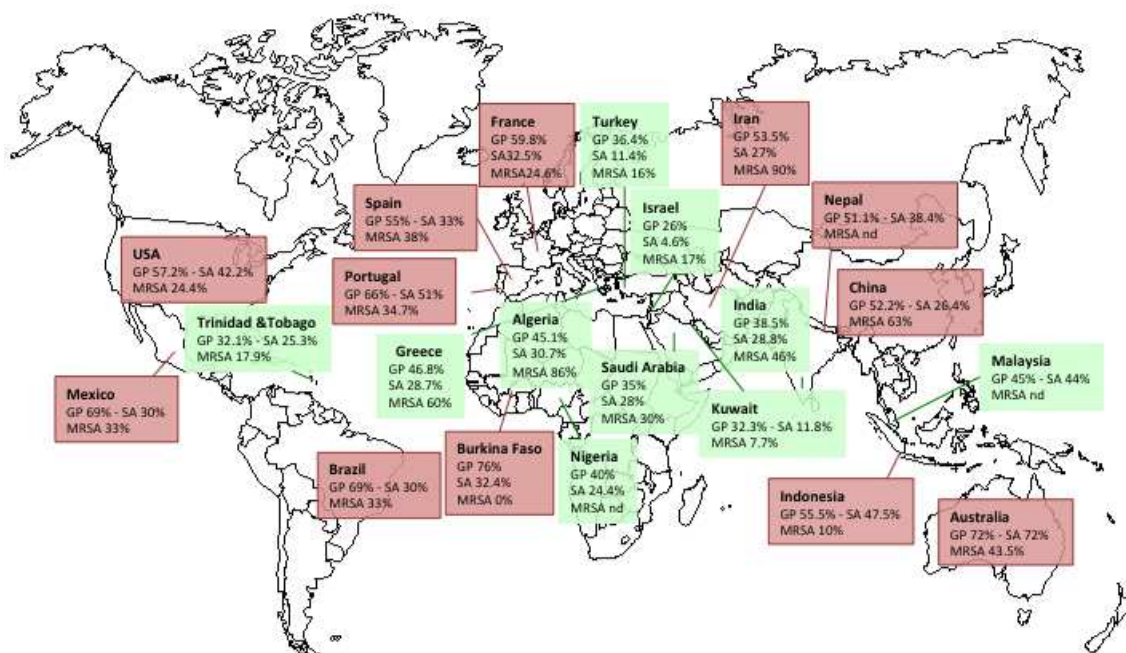


Figure 2. Worldwide geographic distribution of Gram-positive cocci (GP), *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) isolated from diabetic foot ulcers. Red shading indicates regions where GP are predominant. Green shading indicates regions where Gram-negative bacilli are predominant.

2.5. Resistance of *S. aureus* in DFIs

The prevalence of methicillin-resistant *S. aureus* (MRSA) in DFI varies among countries with an exacerbation in countries that are less developed (Figure 2) [19,20,28–30,41–73]. MRSA are more often isolated from patients who have been previously hospitalized or reside in a chronic care facility, who have recently received antibiotic therapy or who have had a previous amputation [41,74].

In France, the prevalence of MRSA increased since the late 1990s [73,75]. Around 2005, different protocols were developed. National guidelines were implemented for the better management of DFI, notably concerning the debridement procedures, the microbiological samplings and antibiotic use [76]. The results of these guidelines entailed a significant decrease in the number of bacteria isolated per sample, in the increased rate of Gram-positive cocci and in the prevalence rate of multidrug-resistant bacteria, notably MRSA [3,15,28,77]. Concomitantly, hospital infection control measures have been improved [42,78,79], notably on the use of hydro-alcoholic solution for handwashing [80]. These measures have been associated with a reduction in MRSA diffusion [41].

For some authors, the isolation of MRSA in DFIs would be associated with more severe infections. However, different articles showed a similar clinical presentation and outcomes between MRSA and other pathogens [81–83].

Finally, some cases of DFIs due to vancomycin-resistant *S. aureus* have been described [84,85]. This type of resistance remains uncommon.

2.6. Pathogenesis

The pathogenesis of *S. aureus* in DFI is classical and corresponds to the physiopathology of skin and soft tissue infection (SSTI) [24,86]. The first defense against *S. aureus* infection is the neutrophil response. When *S. aureus* enters the injured skin, neutrophils and macrophages migrate to the site of infection. *S. aureus* evades this response using different methods (e.g., blocking sequestering host antibodies, chemo-

taxis of leukocytes, hiding from detection via capsule or biofilm formation and resisting destruction after ingestion by phagocytes).

The knowledge of *S. aureus* pathogenicity reveals that these bacteria seem to be adapted for soft tissue and bone infections. Indeed the majority of infections remain localized to the feet. Generally, systemic infection secondary to diabetic foot is less prevalent (around 10%). This becomes particularly noticeable when analyzing the infection process [87]. The first event at the beginning of DFI is the adhesion to surface components (fibrinogen, fibronectin and epidermal keratinocytes). *S. aureus* attachment to ulcer surface depends on bacterial expression of numerous surface proteins that mediate adherence to components of bone matrix and collagen [88]. These bacterial cell surface receptors correspond to adhesins or microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [89–92] (Figure 3). MSCRAMMs facilitate bacterial adhesion to skin tissue. Moreover adhesins are essential in intracellular bone invasion. Indeed, *S. aureus* can invade osteoblasts [93], fibroblasts and endothelial cells. In the intracellular compartment this bacterium forms small-colony variants (SCVs) [94]. Thus, they are able to survive in a metabolically inactive state while preserving the integrity of the host cell. SCVs possess important metabolic and phenotypic differences from ordinary *S. aureus* isolates [94–96]. Indeed, they are relatively resistant to antibiotics [97,98] and, hence, difficult to eradicate with antibiotic therapy [99].

Moreover, the synthesis and secretion of glycocalyx play a role in the virulence of *S. aureus*. This is also documented for strains obtained from DFI [100]. The polysaccharide production begins immediately after the adhesion and covers the bacteria, representing an essential component for the development of a 'biofilm' (Figure 3) [101–104].

In addition to specific adherence mechanisms, *S. aureus* have a number of other virulence factors involved in the infection of soft tissues and bones. *S. aureus* is able to secrete toxins, which can lead to tissue necrosis (Figure 3). Toxins produced by *S. aureus* have an important role in the deepening and spread of the infection in the patient with DFI.

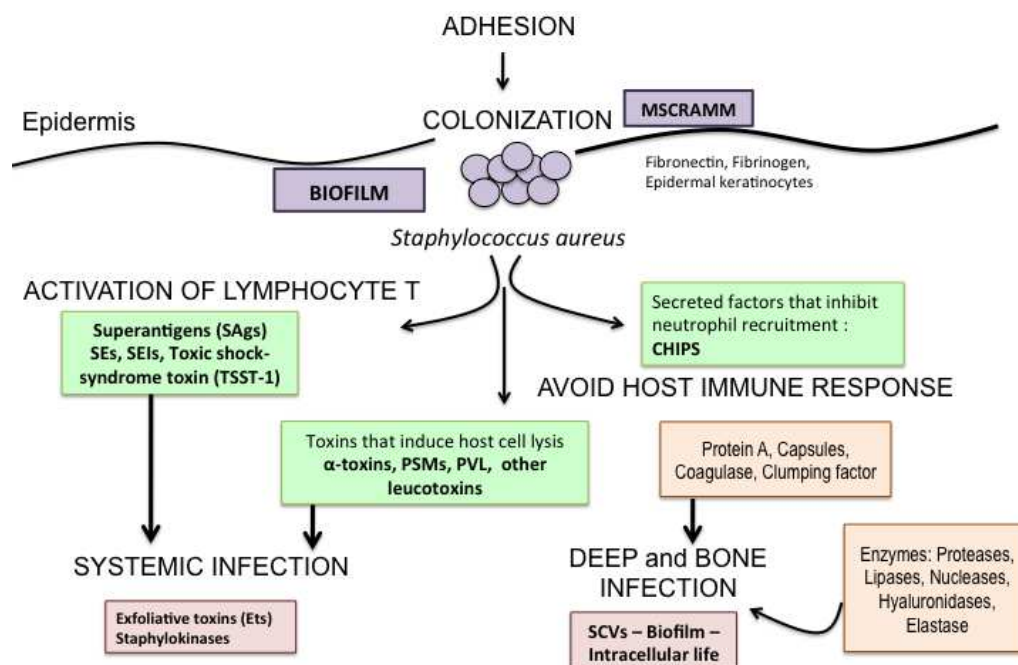


Figure 3. Infection process and virulence factors of *Staphylococcus aureus* in diabetic foot infection; MSCRAMM: microbial surface components recognizing adhesive matrix molecules. *S. aureus* secretes several virulence factors that evade host immune defenses. α -hemolysin (or α -toxin), phenol-soluble modulins (PSMs) and Pantone-Valentine leukocidin (PVL) have the capacity to lyse host cells, which is a mechanism

to evade immune responses. In addition, *S. aureus* secretes factors that inhibit neutrophil function, such as chemotaxis inhibitory protein of staphylococci (CHIPS). In addition, *S. aureus* possesses factors that activate lymphocyte T cells: the superantigens (SE: enterotoxins; SEI: enterotoxin-like protein; TSST: toxic shock-syndrome toxin). On the other side, exfoliative toxins (Ets) facilitate bacterial skin invasion.

3. *Staphylococcus aureus* Toxins in DFIs

The ability of *S. aureus* to cause DFI is defined by numerous virulence factors among which secreted toxins play an important role (participation in colonization, persistence, evasion of the immune system and dissemination) [105]. These toxins include: the pore-forming toxins, the exfoliatins, the superantigen exotoxins (SAG) and the EDIN (epidermal cell differentiation inhibitors) toxins. These cytolytic toxins can damage membranes of host cells leading to cell lysis [106]. Hemolysins lyse red blood cells, while leukotoxins target white blood cells.

3.1. Pore-Forming Toxins

Pore-forming toxins (PFT) of *S. aureus*, through pore-forming and pro-inflammatory activities, have the ability to lyse host cells. They include the single-component α -toxin (or α -hemolysin), the phenol-soluble modulins (PSMs) and bi-component leukotoxins, including Pantone-Valentine leukocidin (PVL), γ -hemolysin and leukocidin D/E [86].

3.1.1. α -Toxin

This PFT is a beta-barrel forming toxin, which consists of beta sheets [107]. It is released by the majority of *S. aureus* as a water-soluble monomer [108]. Its targets are red blood cells and leukocytes except neutrophils [109]. Although α -toxin is the most frequently secreted, few studies have focused on the role of this hemolysin produced by *S. aureus* in DFI. In a French national study, almost all of the strains harbored the α -toxin-encoding gene *hla* independently of the grade [110]. However, this proportion varies between methicillin-susceptible *S. aureus* (MSSA) and MRSA. The α -hemolysin gene was significantly less present in MRSA (16.4%) than in MSSA strains (100%) [48]. As we noted previously, DFIs caused by MRSA present a similar severity of infections to MSSA, excluding an increased role of α -toxin in the pathogenicity of MSSA.

3.1.2. Phenol Soluble Modulins

Recently, the role of a family of secreted peptides, the phenol-soluble modulins (PSMs), has been described in staphylococcal pathogenesis [111]. PSMs are produced by the majority of *S. aureus* strains [112]. These toxins are membrane-injuring toxins. They are structurally characterized as a family of seven small amphipathic α -helical peptides. Some PSMs are described: PSM α 1–PSM α 4 and delta-toxin. Like LukAB (described below), they induce human neutrophil lysis after phagocytosis, a pathogenesis mechanism of great importance for the high toxicity [113,114]. In DFI, to date, no report has evaluated the significance of these virulence factors in the pathogenicity of *S. aureus*.

3.1.3. The Bi-Component Leukotoxins

S. aureus produces some bi-component toxins structurally similar to α -toxin. These toxins result from the association of the class S (Slow) component and the class F (Fast) component based on their electrophoretic mobility [105]. They induce the activation and the permeability of the target cells. They can lyse phagocytes (monocytes-macrophages and neutrophils), which is considered important for *S. aureus* immune evasion [115,116]. These PFTs include: (i) the gamma-toxin (gamma-hemolysins HlgA and

HlgC/HlgB); (ii) the Pantone-Valentine leukocidin (PVL) [117], corresponding to the LukS-PV and LukF-PV proteins; (iii) the leukocidins LukDE [118,119]; and LukAB [116] (also known as LukGH) [116].

The *hlg* gene cluster encoding for hemolysin- γ (Hlg) and hemolysin- γ_2 (Hlg2) is located in the core genome. This cluster is present in almost all *S. aureus* strains. These toxins play a role in septic arthritis and could help community-acquired MRSA (CA-MRSA) to survive in human blood during infection [120,121]. In DFI, all of the isolated strains possess the different components of the *hlg* gene [48,110]. Interestingly, a variant of *hlg* (*hlgv*) is significantly associated with strains isolated from infected ulcers (Grades 2–4) [110,122].

The most well-known leukotoxin is PVL. PVL confers cytotoxicity on neutrophils and monocytes-macrophages, leading to a high virulence [123]. LukS-/LukF-PV are encoded within lysogenic phages [124,125]. Several *lukS-/lukF-PV*-transducing phages have been discovered [105]. This particular genetic organization involves an easy horizontal transmission of PVL genes in *Staphylococcus* spp. [126].

The PVL-positive strains are responsible for SSTIs (abscesses, furuncles, carbuncles or necrotizing fasciitis), severe necrotizing pneumonia and aggressive bone and joint infections [48,105,127,128]. However their prevalence is extremely diverse, varying between less than 5% and 67% of MSSA [129–136]. Interestingly, PVL-positive strains are statistically associated with younger patients [137]. This toxin has been linked to CA-MRSA infections [129], even if some CA-MRSA isolates do not carry the *lukS/lukF-PV* genes [138]. A high prevalence of CA-MRSA was observed in Africa [139], the Middle East [140,141], Asia [142] and America [143].

The PVL-producing clones were rarely isolated from DFI (Table 2) [48,110,122,144,145]. However, this prevalence varies between countries: France (~3%), Algeria and The Netherlands (~14%) [48,146]. The main PVL-producing strains isolated from DFI belong to ST80-MRSA (14/21 strains), followed by ST152 (6/21) and CC30 (1/21). ST80 is the main PVL clone circulating in Europe and North Africa [147–149]. Rates of PVL-producing clones in DFI remain low in comparison to data concerning SSTIs [127] (e.g., the epidemic furuncles (42%), major abscesses (73%) and gold surgically-drained abscesses (89%)) [146,150–152]. Indeed, in DFI, a higher incidence of PVL-positive isolates among subjects with CA-MRSA could be observed (31.8% versus 5.7%; $p = 0.004$) [153].

The role of PVL in DFI remains under debate. The different PVL clones are equally distributed among each grade [122]. The majority of Grade 1 ulcers where PVL-positive strains were isolated had a rapid amelioration [122], and for instance, in Algeria, all of the patients harboring PVL-positive strains isolated from DFI had a worsening evolution. However, the management of chronic wound infections in this country is clearly different from international recommendations, and the use of amputations is frequent, independent of the evolution of the wound. Finally, it is interesting to note that strains isolated from DFOM harbor neither *lukF-* nor *lukS-PV* [154]. Thus PVL-positive strains are scarce in wound ulcers, and their pathogenicity is not clearly established.

Table 2. Repartition of toxin-encoding genes in *Staphylococcus aureus* isolated from diabetic foot ulcers at different clinical grades.

Genes	PEDIS Grades			Total	References
	Grade 1 <i>n</i> = 99	Grade 2–3 <i>n</i> = 481	Grade 4 <i>n</i> = 94		
<i>lukF/lukS-PV</i>	3 (3%)	15 (3.1%)	3 (3.2%)	21 (3.1%)	[48,110,122,145]
<i>edin</i>	6 (6.1%)	12 (2.5%)	10 (10.6%)	28 (4.1%)	[48,110,122,145]
<i>tsst</i>	5 (5.1%)	40 (8.3%)	12 (12.8%)	57 (8.5%)	[48,110,122,145]
<i>etA, etB, etD</i>	4 (4.0%)	17 (3.5%)	13 (13.8%)	34 (5.0%)	[48,110,122,145]
<i>sea</i>	39 (39.4%)	201 (41.8%)	39 (41.5%)	279 (41.4%)	[48,110,122,145]
<i>lukDE</i>	66 (66.7%)	263 (54.7%)	49 (52.1%)	378 (56.1%)	[48,110,122,145]

LukED exhibits toxicity toward PMNs in vitro and induces dermatonecrosis when purified toxin is injected into rabbits [118,119]. Moreover, this toxin plays a critical role in *S. aureus* lethality for mice. It targets and kills murine phagocytes (monocytes-macrophages and neutrophils), promoting disease progression [155]. In uninfected DFU, LukED is equally distributed among grades (52%–66%) when we pooled all of the data (Table 2). However, when data are analyzed separately, we showed that the *lukDE* gene was significantly more often associated with strains isolated from infected ulcers (Grades 2–4) [122]. In a more recent study, this gene was clearly identified as a marker that differentiated uninfected from infected ulcers and predicted the outcome of Grade 1 DFU [110]. The association between *lukDE* and MRSA has been also reported in DFI [48,156]. In DFOM, this gene was present in approximately 40% of strains [154]. However, even if experimental analysis showed the virulence potential of this leukotoxin, clinically, it seems to present a poorer activity compared to PVL, and the reduced virulence observed may be a response to atypical local inflammatory reaction [156].

The last leukocidin characterized is LukAB/HG. This new member of the *S. aureus* leukotoxin family contributes to neutrophil killing, promotes the survival of *S. aureus* in human whole blood, restricts neutrophil-mediated killing and promotes CA-MRSA pathogenesis [115,116]. No data concerning its implication in DFU have been reported.

3.2. Exfoliative Toxins

Exfoliative toxins are serine proteases secreted by *S. aureus*. Three (ETA, ETB and ETD) out of the four different serotypes of this toxin are linked to human infection. The exfoliatins act as “molecular scissors” facilitating bacterial skin invasion [157]. The prevalence of *eta* and/or *etb* ranges from 0.5%–3% in MSSA [157–159], whereas around 10% of MRSA are *eta*-positive [159].

In uninfected DFU, the distribution varies among clinical grades (Table 2). Interestingly, strains harboring these genes are three-times more frequent in Grade 4 (13.8%) as compared to Grade 1 (4%) or Grades 2–3 (3.5%). However, each exfoliatin does not have the same representation: if *eta* and *etb* are rare (1.3%) or absent, respectively, *etd* is the most prevalent (3.7%), particularly in strains isolated from Grade 4 (10.6%). In a previous study, we could also note that two of four patients harboring an *etd*-positive strain present on a Grade 1 ulcer had a worsening evolution [17]. Post et al. showed an important presence of *eta* (13%) and *etb* (17%) in DFI (no screening of *etd* was noted) [144]. However, as no grade has been reported in this work, it is not possible to link exfoliative genes and the severity of the DFI. Finally, this gene was absent in strains isolated from DFOM [154].

3.3. Enterotoxins

Enterotoxins are secreted toxins of ~20–30 kD that belong to the family of superantigens (SAg). These molecules over-induce cytokine production from both T-lymphocytes and macrophages [107]. The mechanisms by which staphylococcal enterotoxins work are not well known, but may include the activation of cytokine release, ultimately causing cell death by apoptosis. They contribute significantly to major illnesses [160,161]. A recent classification distinguishes three groups of SAg: staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like toxins (SEls) and toxic shock-syndrome toxin 1 (TSST-1) [162].

3.3.1. Staphylococcal Enterotoxins and Enterotoxin-Like Toxins

The majority of *S. aureus* isolated from DFU have the capacity to produce a large number of Sags, notably SEs and SEls [161]. These toxins activate T cells, resulting in a high secretion of proinflammatory cytokines. This process leads to a chronic inflammatory state in uninfected DFUs, inducing a delay or an absence of wound healing [163]. Genes, including the *sea*, *sed*, *seg* and *sei* genes, code for enterotoxins found in *S. aureus* isolated from DFI. We observed that *sea* and *sei* are significantly more prevalent in Grade 2–4 ulcers than in Grade 1 (Table 2) [122] and could represent a biomarker to differentiate infection

and colonization. The majority of enterotoxins are more frequently identified in MRSA strains except for *seb* and *seh* genes [122]. One of the main enterotoxins is SED, present in around 40% of the strains [122,161]. The *sed* gene is often located on a plasmid, and the active protein is structurally similar to SEA [164]. SEA could have a major role in atopic dermatitis by inducing the upregulation of adhesion molecules and eliciting inflammatory responses in endothelial cells and keratinocytes [165]. Thus, SED may be selected in DFU isolates because, similar to SEA, it has an enhanced ability to induce local inflammatory responses.

3.3.2. Toxic Shock-Syndrome Toxin 1

The best known *S. aureus* superantigen is the 22-kD toxic shock-syndrome toxin 1 (TSST-1), which causes toxic shock syndrome (TSS). Additionally, SEI-X is a new member of the *S. aureus* SAg family, and it has been shown to have an important role in *S. aureus* necrotizing pneumonia infection caused by USA300 MRSA strain [166]. Vu et al. found that 88% of the DFU isolates carried the gene for SEI-X; the remainder contained the gene for TSST-1, and one isolate had genes for both SEI-X and TSST-1. Typically, *S. aureus* strains have the gene for either SEI-X or TSST-1 [161]. The prevalence of the *tst* gene is low in the strains isolated from diabetic foot (~8%). However, as we observed for *etD*, this gene is more frequently present in Grade 4 (Table 2) and absent in DFOM [154].

3.4. Epidermal Cell Differentiation Inhibitors Toxins

EDINs toxins are members of a group of major bacterial virulence factors targeting host Rho GTPases [167]. Recent findings suggest that EDIN toxins might favor bacterial dissemination in tissues by a hematogenous route, through the induction of large transcellular tunnels in endothelial cells named macroapertures [168–170]. Indeed, recent data showed that EDIN toxins promote the formation of infection foci in a mouse model of bacteremia [171]. To date, three isoforms of EDIN have been characterized: the first discovered EDIN isoform (EDIN-A), isolated from the E-1 strain of *S. aureus* [172], as well as EDIN-B [173,174] and EDIN-C [175]. A first epidemiological survey, involving staphylococcal strains isolated from patients hospitalized for various infectious diseases, demonstrated a higher prevalence of *edin*-encoding genes in this group compared to nasal strains isolated from healthy patients [176]. Munro et al. showed that 90% of all *edin*-bearing *S. aureus* isolates carry the type-C allele. These isolates are more significantly associated with deep-seated soft tissue infections than other types of infections [177].

Messad et al. analyzed the distribution of *edin* genes in *S. aureus* isolated from DFI in a French national collection. *edin-B* is the most prevalent *edin* gene associated with DFIs (Table 2) [17]. The clonal complex analysis indicated that *edin*-positive strains belonged to four major groups: a singleton near CC8 (*edin-A*), a singleton belonging to ST152-MSSA (*edin-B*), CC80-MRSA (*edin-B*) and the most prevalent CC25/28-MSSA (*edin-B*). The distribution of *edin* genes in DFI shows an important presence of these genes in strains isolated from Grade 4 ulcers (2.5% for Grades 2–3 vs. 10.6% for Grade 4). Of note, patients with Grade 1 ulcers that presented *edin*-positive strains had a worsening evolution [17]. However, these genes seem to be absent in strains isolated from DFOM [154]. These observations support the idea that EDIN might work together with the arsenal of *S. aureus* virulence factors to give the bacteria a higher potential for systemic infection [168]. *edin* encoding genes thus represent additional markers of interest to differentiate infecting from colonizing *S. aureus* strains in DFU and to predict the wound outcome.

4. Conclusions

In conclusion, DFI is a complex pathology involving the virulence of bacteria and host responses. Indeed, its main feature is the coexistence of multiple bacterial species on the chronic wound organized in pathogroups and the host-related responses encountered by bacteria, which modify the bacterial pathogenicity. Some studies showed the presence of toxinogenic *S. aureus* strains (harboring exfoliatins-, EDIN-,

PVL- or TSST-encoding genes) in DFI, notably in Grade 4, with systemic impact. The absence of the same strains in DFOM suggests that these strains may not be adapted to colonize wounds. On the other hand, the main population of *S. aureus* (non-toxinogenic) isolated in uninfected DFU is perfectly adapted to infect deep structures and bone. The rarity of systemic cases (Grade 4) and of the presence of toxinogenic bacteria would, by definition, be an infected wound. However, it is interesting to note that some toxinogenic strains with a high pathogenic potential are present on uninfected wounds promoting no virulence in this polymicrobial environment. Moreover, no clear link between these strains and amputations has been noted to date. Subsequent studies are required to understand the role of toxins and their real impact in DFU. Screening the presence of genes encoding toxins by molecular biology tests on uninfected DFU could also represent a new approach for patients for whom the clinical diagnosis of infection is hampered by peripheral arterial disease, neuropathy or impaired leukocyte functions in the aim to predict if *S. aureus* is going to be invasive or not (needing an antibiotic treatment).

Acknowledgments: U1047 Team was supported by INSERM (Institut National de la Santé et de la Recherche Médicale).

Author Contributions: C.D.-R., C.N.E., A.S. and J.-P.L wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Facteurs qui inhibent les espèces réactives de l'oxygène

S. aureus produit également des facteurs qui inhibent les espèces réactives de l'oxygène (ROS) induisant la mort par les polynucléaires neutrophiles, comme le pigment caroténoïde de *S. aureus* et les enzymes de la superoxyde dismutase (Figure 6) ⁴².

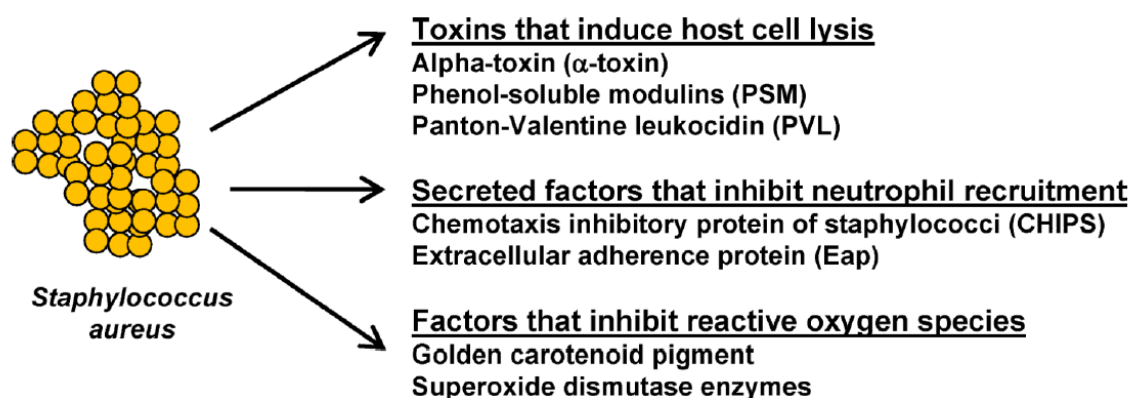


Figure 6: Facteurs de virulence de *S. aureus* durant l'infection cutanée ⁴².

4. Régulateurs des facteurs de virulence

L'ensemble des facteurs de virulence sont sous la dépendance d'un réseau global de régulateurs complexe qui interagissent ensemble et coordonnent l'expression des différents facteurs de virulence très importants pour l'établissement de la pathogénicité de *S. aureus* ((cf Figure 4 et article 2 : Complex network of virulence genes regulators in *S. aureus*). Ce sont : Agr (régulateur global), Sar (Staphylococcal Accessory Regulator), Sae (*S. aureus* exoprotein expression), Rot (Repressor Of Toxin), sigB (sigma B) ⁵³⁻⁵⁷, ArIRS (Autolysis-Related Locus), SrrAB (Staphylococcal Respiratory Response Systems) ^{54,57-61}, LytRS ^{59,60}, TcaR (Teicoplanin-associated locus regulator) ^{60,62}, Mgr (multiple gene regulator) ^{54,57,61}, svrA (staphylococcal virulence regulator) ^{53,55}, vraSR (vancomycin resistance associated sensor/regulator) ^{63,64}, Fur (Ferric Uptake Repressor) ^{41,44,59}, GraRS (glycopeptide resistance-associated) ^{65,66}.

Travail n°2

Complex network of virulence genes regulators in *S. aureus*

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Revue : Article en préparation

Complex network of virulence genes regulators in *Staphylococcus aureus*

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Keywords: *Staphylococcus aureus*, virulence genes, regulators.



Introduction

Staphylococcus aureus is a Gram positive cocci, facultative aero-anaerobic which is able to cause a wide range of diseases from skin and soft tissue infections (e.g. impetigo, carbuncles) to life-threatening bacteraemia, toxic shock syndrome, endocarditis, and osteomyelitis ^{1,2}. To carry the infection, *S. aureus* deployed an arsenal of virulence factors to colonize, invade and destroy host immune cells and tissues. Classically the proteins that contribute to the virulence of *S. aureus* are divided into five major families: adhesion proteins, superantigens, pore-forming toxins, ADP-ribosylating toxins and proteases.

Those virulence factors are adhesins or MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that bind to different host proteins. The MSCRAMMs include fibronectin binding proteins (FnBPs), fibrinogen and collagen and extracellular matrix binding proteins (*eap*, *emp*) which are important for adhesion and colonization ³⁻⁵. Toxins and exoproteins are also used to destroy host tissues and to allow the *S. aureus* dissemination. The main toxins produced are alpha-hemolysin (Hla), gamma-toxin (gamma-hemolysin, HlgA, HlgB, HlgC)⁶, Panton-Valentine leukocidin (PVL) that are pore-forming toxins ⁷⁻⁹, exoenzymes EDINs (epidermal cell differentiation inhibitor -A, -B and -C) which inactivate the small GTPase RhoA, to promote the dissemination of the bacteria in blood stream and various host tissues ^{10,11}. Different enzymes (e.g. proteases, nucleases, hyaluronidases, lipases) ¹², capsules and protein A (*spa*) are also produced to escape to immune system and protect against host-mediated clearance ¹³. In addition, other virulence factors are secreted by *S. aureus* to inhibit neutrophil recruitment such as chemotaxis inhibitory protein of staphylococci (CHIPS), to activate lymphocyte T cells such as the superantigens (SE: enterotoxins; SEI: enterotoxin-like protein; TSST: toxic shock-syndrome toxin). Exfoliative toxins (Ets) are used to facilitate bacterial skin invasion ¹⁴.

Virulence factor expression appears to be controlled by the concentration of autoinducing peptides and by bacterial density, pH and CO₂, and each of these signals controls different regulatory systems. In fact many regulators interact together in a complex network to coordinate temporal expression of virulence factors that play a role in the pathogenicity. Therefore, determining the structure and target genes of all these regulators represent a major interest to develop new therapeutic strategies to fight against these bacteria during infection that why many studies were done on it.

The main regulators identified are Agr (accessory gene regulator), Sar (staphylococcal accessory regulator) family⁵, Sae (*S. aureus* exoprotein expression) ¹⁵⁻¹⁹, SigB (sigma B)¹⁷, ArlRS (autolysis-related locus), SrrAB (staphylococcal respiratory response systems)^{5,15-21}, LytRS ^{5,19,21,22}, TcaR (Teicoplanin-associated locus regulator)^{5,16}, SvrA (staphylococcal virulence regulator) ^{23,24}, VraSR (vancomycin resistance associated sensor/regulator)^{15,17,21,25}, Fur (ferric uptake repressor) ^{2,6,19}, WalKR ^{22,26}, GraRS (glycopeptide resistance-associated) ^{22,25,26}, HssRS (heme sensor system) ²⁵, AirRS, and BraRS (Bacitracin resistance associated) ²⁶.

Many studies were previously done to determine the structure and to understand the mechanism of action of those regulators and the impact of those regulators on virulence genes. Informations were synthesized about structure of each regulator, virulence genes impacted by those genes and relation between all those regulators.

The global regulator Agr

The *agr* system is the best characterized among *S. aureus* regulators. It is an autoinducing quorum-sensing system that influences the overall regulatory network and it plays an important role in pathogenesis.

Agr consists of two divergent transcripts, RNAII and RNAIII^{27–32}. RNAII is a set of four genes *AgrBDCA*^{27–32} that work together under the dependence of the promoter P2. AgrD, with the assistance of the membrane-associated protease (AgrB), leads to the synthesis of the autoinducing peptide (AIP)^{33,34}. At critical level, AgrD is recognised by AgrC, the sensor, which leads to the activation by phosphorylation of the cytoplasmic regulator AgrA^{35,36}, which will affect directly or indirectly the target genes. AgrA also has a positive feedback on the production of the four genes *AgrBDCA* by inducing upregulation of RNAII after fixation on the promoter P2¹⁹ (Figure 1).

The second promoter P3 can induce the transcription of RNAIII^{4,29,35}, the effector molecule of the *agr* system^{28,37–39} which expression is maximal during transition of the post-exponential to the stationary phase^{4,11,40}.

It has been shown that *agr* system can influence the regulation of several virulence genes of *S. aureus*. According to Dunman *et al.*⁴¹, it can up-regulate 104 genes and down-regulate 34 genes in a cell density dependent manner¹⁹. Many studies have shown that the activation of the effector molecule (Agr RNAIII), leads to upregulation during later stage of growth⁴ or postexponential phase¹⁹ of several secreted proteins or exotoxins^{4,5,11,19,21,42–46}: alpha- and beta-toxins^{19,21,45,46}, delta-gamma hemolysin¹⁹, lipases, proteases⁴⁵, TSST-1^{19,47}, PVL^{8,20,35}, leukotoxins²⁴ as lukED (leukotoxin ED), enterotoxins BCD, exfoliatin toxin A and B (EtAB), capsular polysaccharide (cap5, cap8)¹⁹.

On the other hand, *agr* system can repress the transcription of selected genes encoding cell surface proteins^{4,5,11,19,36,42–44} such as Spa, fibronectin-binding proteins^{19,21,45,46}, coagulase⁴⁵.

agr system can initiate or repress transcription of all these genes by acting directly on the promoter target genes or indirectly by influence of regulators¹⁹. For example, SarS activates *spa* by binding its promoter and it has been shown that Agr can downregulate *sarS* expression and thus downregulate *spa* expression⁴⁵. Agr/RNAIII can activate the transcription of *kdpDE* (potassium transport system), which is regulator of colonization and invasion factors such as Spa, Cap, Hla, Aur (aureolysin), Geh (lipase) and HlgB²⁵. The repressor of toxins Rot is repressed by *agr* system³⁸. MgrA (multiple gene regulator) can also be target by Agr³⁸.

By inducing activation of exotoxins, *agr* system is therefore necessary in acute phase of the infection to destroy host tissues and fight against immune cells⁴⁸. On the contrary, during chronic infection, repression of *agr* is necessary to allow bacteria to persist without provoking or destroying host cells⁴⁸. The downregulation of *agr* will be necessary to promote biofilm formation^{42,49}, important for persistence. So, Agr can induce up or down-regulation of virulence genes in acute infection but when necessary as in chronic infection it should not have any effect. This regulation of *agr* system can be done by itself as previously published that *agr* system can be auto-regulated by *agrA*⁵, but *agr* system can also be regulated by other genes or other factors. These factors are SigB and SarX (member of SarA family proteins) which can negatively regulate *agr* while *norG*⁵⁰ (murein hydrolase)⁵¹ can upregulate *agr* expression. At low oxygen condition, *agr* expression can be upregulated by *SrrAB*. *agr* system can also be upregulated by other Sar family regulators such as SarA and SarR at early growth phase and by MgrA, SarX and SarZ to the stationary phases⁵.

The staphylococcal accessory regulator (Sar)

The *S. aureus* genome encodes a number of transcriptional factors. Sar is a family of proteins ⁵² that shares sequence homology ⁵³. It includes: SarA, SarR, SarS, SarT, SarV, Rot ^{52,54}, SarU, MgrA ²⁷, SarX, SarZ ^{16,50} and SarY ²⁹.

➤ SarA

SarA is the major protein encoded by the *sar*-locus ⁴⁹ and the first characterized member of the SarA protein family ^{16,54}. *sarA* locus is composed by three promoters (P2, P3, P1)⁵⁵ that yield three overlapping transcripts (*SarA* P2, P3, and P1 transcripts) ^{21,29,56–58} or named (SarA, SarC, SarB) ^{4,36,46}, all encoding for SarA protein ^{36,57}. Activation of *sarA* P1 and P2 is SigA dependant and mostly occurs during exponential phase while activation of *sarA* P3 occurs during postexponential phase or during metabolic stress periods and is SigB dependant ^{46,56,57}. Sar proteins can be also downregulated by SarR that can bind to the upstream promoter region of the P1 *sarA* ^{5,59} and some studies demonstrated that *sarA* locus may be autoregulatory ⁶⁰.

Previous studies have demonstrated that SarA can control more than 120 genes ^{54,56,61} directly by binding to promoter regions or indirectly by affecting other regulators ⁵.

Directly, SarA has been reported to upregulate transcription level of adhesion genes (fibronectin-binding protein A FnbA ^{5,36,55,58,62}, FnbB ¹⁹), toxins (alpha, beta and delta ^{5,37,55}, gamma Hemolysin) ¹⁹, PVL ^{19,20}, LukED, enterotoxins, TSST-1 toxin and capsule biosynthesis genes ^{5,19}, Ica ^{63,64}, EDIN-B ¹¹, secreted proteins, Eap and Emp ⁶⁵.

SarA induces also downregulation of several other genes such as serine protease (*ssp*) ³⁷, collagen adhesion (*cna*) ^{19,58}, aureolysin (*aur*), *spa* ^{5,55,58,62,66,67} and gene encoding for collagen binding protein ⁵ that are important for adhesion, escape, cell wall metabolism and autolysis. Super-oxide dismutase and thioredoxin reductase transcription involved in oxidative stress response are also repressed by SarA ⁶⁸.

Indirectly, SarA can have an effect on numerous genes by activation of the *agr* system ^{56,66,69} in early phase of growth ⁵. SarA binds to *agr* P2 and to a lesser extent, P3 promoter leading to activation of RNAII and RNAIII ^{27,36,69,70}. Manna and Ray ⁵⁴ indicated that SarA is involved in negative regulation of *rot* transcription during the postexponential phase of growth ²⁰. SarA can also downregulate *sarT* ^{55,58,66}, repressor of *hla* ⁵⁸. Moreover, SarA is also required for *saeRS* and *arlRS* activation ⁵⁸ and can also target other promoter regions of *sarS*, *rot*, *sarV*, *sarT*, *icaRA* (Intercellular Adhesion) ⁵.

➤ The repressor of toxins Rot

Another best-characterized of the Sar family member is the repressor of toxin (Rot) ^{71–73}. It's a likely a winged-helix protein with 133-residue SarA homolog ^{55,72}.

Rot is a global regulator in *S. aureus* that can positively or negatively modulate virulence-gene expression ^{45,53,72}. It has been shown by transcriptional analysis that Rot affects the transcription of 168 genes ^{53,55}.

It can negatively regulate some genes encoding various secreted exoproteins ^{20,52,73} during exponential phase of growth such as lipase, toxins ⁷²(hemolysins ^{45,52,71,74}, PVL ^{20,71}, other pore-forming toxins) and proteases ^{45,52,71,74} which play a role in tissue invasion ^{45,74}.

However, Rot can also upregulate genes encoding cell surface proteins ^{45,53,74,75} including protein A ^{71,75}, fibrinogen, and fibronectin binding proteins, superantigen-like exoproteins (Ssl7) ⁷¹, SdrC (cell surface adhesion) ^{55,75} and ClfB (clumping factor B) ⁷⁵.

Rot can be a positive regulator of *sarS* expression and thus, indirectly, *spa*^{45,66}, *clfB*, and *sdrC*⁵² expression. Rot and SaeRS can cooperatively activate expression of the staphylococcal superantigen-like exoproteins⁵³. It was shown that *rot* is positively auto-regulated⁵² while it's negatively regulated by *agr* system^{52,76}.

➤ The multiple gene regulator MgrA

MgrA^{39,77,78} that is also named Rat (regulator of autolytic activity) or NorR⁷⁸ is a 147-residue protein^{78,79} that belongs to the SarA family⁷⁷ and has homology with SarA^{21,78}, MarR^{21,78,80,81} member's families and also MgrH1 (MgrA Homologue 1) also known as SarZ⁸². It contains a DNA-binding helix-turn-helix motif^{39,68,80,81,83}, a dimerization domain and a single cysteine *cys12*^{68,79}. Two promoters can transcribe *mgrA*^{38,78,84,85}: P2 (302 nucleotides) and P1 (124 nucleotides)⁸⁵. Ingavale *et al.* showed a constitutive expression of both promoters in all growth phase in cells⁸¹.

It was previously showed that MgrA can be repressor or activator of multiple virulence genes in *S.aureus*⁸⁶. Microarray performed by Luong *et al.*⁸³ showed 175 genes upregulated by MgrA, and 180 genes downregulated. These genes are involved in different parthways (figure 2).

MgrA is a negative^{51,51,68,81,83,84,87} and important regulator of autolytic activities^{21,83,88,89}. Crosby *et al.* demonstrated that mutants *mgrA* showed an increase of biofilm formation⁸⁵ by controlling regulation of surface proteins^{77,85} and it has a role in adhesion by regulating capsule biosynthesis^{21,68,77,83-85} and cell surface proteins⁸⁵. MgrA affects leukotoxins⁷⁷, leukocidin⁸³, α -toxin^{21,77}, Exotoxins, CHIPS⁸⁵ and Spa^{21,68,83,85,88} expression. General metabolism⁸³ and antibiotic resistance^{79,80,86,90} can also be modulated by MgrA. It is involved in oxydative stress⁶⁸.

So, MgrA has a direct effect on *S. aureus* virulence. It can also act on several genes through different regulators^{21,27,77}. But its expression is under regulation of several regulators. *mgrA* expression can be activated by ArlRS⁸⁵, RNAIII^{38,84} and SarZ⁵⁰. RNAIII can lead *mgrA* expression through P2 promoter^{38,84}. On the other hand, *mgrA* can be negatively regulated by SigB⁸⁴ and can be also autoregulated^{81,84}.

External factors can also regulate *mgrA* such as thiol-based oxidation⁸², eukaryotic- like serine/threonine kinase (Stk1 or PknB), RsaA (non-coding RNA)^{38,84}, salicylate⁹⁰, NorA (multidrug efflux pump)⁷⁸, H2O2 and iron through Fur and PerR (Peroxide-responsive repressor)⁶⁸.

➤ SarS or SarH1

SarS, similar to SarA and other homologs, encodes a 250-residue protein^{29,60,67} which can be divided in two homologous halves of 125 residues^{29,60,67,91}, named S1 and S2, respectively²⁹. Each SarS homologous domain contain five alpha-helices and three beta-strands²⁹.

SarS is known to activate Spa synthesis^{29,55,67}. It can also repress *hla*⁵⁵. On the other hand, SarS can regulate *sarA* and *agrA*. Luong *et al.* showed that it activates Spa in an Agr-dependent manner and repress Hla in SarA-dependent maner³⁹.

On the other way, SarS is repressed by Agr, SarA^{29,67} and MgrA⁵⁵ and activated by SarT, TcaR⁸⁹, Rot⁹² and ClpXP protease (intracellular protease)⁵⁵. SigB can also influence *sarS* expression⁸⁹.

➤ SarR

SarR is a part of winged-helix DNA-binding protein family²⁹ that was defined as a 13.6 kDa protein⁶⁰. The overall structure of the SarR monomer consists of five helices, three short β -strands, and several loops⁶⁰. SarR repressed the transcription of autolysis genes *aur* (aureolysin) and *sspA* (serine protease)⁵. It can regulate *agr* expression²⁷ and negatively regulate *sarA* by binding its promoters^{39,60,90}. However, it has been shown that exposition to salicylate can downregulate its expression⁹⁰.

➤ SarX

SarX was defined as a 141-residue polypeptide that shares 54, 51, 49, 51, 46, and 47% homology with SarS, SarV, SarR, SarU, SarA, and Rot, respectively ²⁷. Its expression is maximal during post-exponential growth phase and MgrA is the only one among SarA proteins family which can activate its transcription ²⁷. Expression of *sarX* leads to upregulation of hemolysins such as *hla* and *hly* and proteases *sspA* ²⁷. It can also downregulate *agr* transcription ²⁷.

➤ SarZ

SarZ is a thiol-based oxidation sensing protein ⁸² that has a DNA- binding helix–turn–helix motif, a single cysteine (Cys13), and a dimerization domain like MgrA ⁶⁸. In cells, it has been shown that primarily, it upregulates genes in the exponentielle phase and downregulates some genes in the post-exponential phase ⁸².

It has been shown that it plays different roles in *S.aureus* global regulation: metabolism, antibiotic resistance ⁸², oxidative stress ^{68,82}, autolysis, and virulence ⁸². Chen *et al.* have shown that it can influence expression of 87 genes. More specifically, it can activate expression of genes involved in amino acid metabolisms (*argGH*, *ilvD*, *lysC*), fatty acid synthesis (*fabG*), sugar metabolism (*gntRK*, *lacD*, *malA*, *treC*), pyruvate metabolism (*acs*, *pflAB*, *pckA*), and gluconate metabolisms (*gntR*, *gntR*-like gene) ^{21,82}. However, it induces downregulation of genes involved in pyrimidine and histidine metabolism (*pyrR*, *hisC*) ⁸².

Virulence genes can also be affected. SarZ activates expression of toxin (Hla) ⁹³, cell surface proteins (EpiEF, LrgB) and two drug transporters (NorB, Tet38) important for antibiotics resistance ⁹⁴ while other cell surface proteins were repressed (IsdC, Efb, Fib, TcaA) ⁸².

About *sarZ* regulation, it has been shown that *cvfa* gene, virulence regulatory gene in *S. aureus* can activate its transcription ⁹⁴.

➤ SarT, sarV, sarU

To our knowledge there's no publication that described and studies those regulators. However, Trotonda *et al* demonstrate that *sarV* is a marker of autolysis ⁹⁵. On the other hand, some studies revealed that *sarT* repressed *hla* transcription ^{21,27,55} and *sarU* ⁵² activates *spa* expression by binding *sarS* promoter ^{21,52,55,66}. In turn, *sarT* expression can be promoted by Spa ⁹⁵ and repressed by Agr ⁶⁶ and SarA ⁵⁵.

Moreover, it has been shown that *sarT* and *sarU* are involved in the regulation of *agr* transcription ^{21,39} and SarU positively regulates *agr* expression ⁵².

SigB

SigB is one of the three sigma factors (σ_B , σ_H , and σ_S RNA polymerases) of *S.aureus* ⁹⁶. *sigB* gene is included in four-gene operon ⁴: *rsbU*, *-V*, and *-W* ^{19,58} and *sigB* ⁹⁷. RsbW is an anti-sigma factor ^{4,19,97,98} which regulates SigB activity post-translationally ⁴ by binding SigB and forming a RsbW– SigB complex ¹⁹. RsbU is required for *sigB* activation ⁴ by controlling the availability of free RsbW to interact with SigB through RsbV phosphorylation ⁹⁷.

The activation of *sigB* occurs in stress environmental condition and energy depletion (reduced ATP/ADP ratio) and can be stimulated by environmental stimuli such as ethanol and salicylic acid ⁹⁹.

SigB has been shown to be an important regulator of the general stress ⁹⁶ and heat-shock response ⁴. Moreover, it modulates the expression of at least 30 *S.aureus* genes ¹⁹ involved in stress response, heat,

oxidative ⁴⁹ and antibiotic stresses ^{49,97}, adhesion, inflammation, biofilm and thus has been shown to be important for the bacteria virulence regulation.

SigB factor is associated to downregulation of toxins (Hla) ^{48, 46}, but it induces also upregulation of adhesins as ^{24,48,62} FnbB ⁵⁸, FnbA ⁴⁶, clumping factor ^{24,62} and Cap ²⁴ necessary for early stages of biofilm formation ⁶².

SigB plays also a critical role during persistence by inducing SCVs (Small Variant Colonies) formation under stress conditions ⁴⁸. They are small colonies, metabolically inactive that doesn't induce host immune system response and that are characteristic of *S.aureus* in persistent infection ^{48,49}. This phenotype allows to the bacteria to resist in persistent stress conditions.

Concerning the regulation of other regulators, studies demonstrated that SigB downregulates *agr* system ^{46,49} while it upregulates SarA expression ⁴⁹.

***Staphylococcus aureus* exoprotein (Sae)**

Four ORFs composed *sae* locus: ORF1 (*saeR*) encoding for the response regulators and ORF2 (*saeS*) encoding for a sensor kinase with strong homology to histidine kinase ^{4,17,72,100}. ORF3 (*saeQ*) encoding a membrane protein involved in the stabilization of SaeS ⁷³, ORF4 (*saeP*) encoding a putative lipoprotein ^{15,73,100}. *SaeP* and *saeQ* are localized upstream of *saeRS* and are important for operation of the *sae* operon ^{24,101,102}. The response regulator SaeR, belongs to OmpR/PhoB subfamily of response regulators. It consists to N-terminal receiver domain and a C-terminal DNA-binding domain (DBD) ¹⁰³.

The locus *sae* has two promoters: P1 and P3 ^{15,17,73}. P1 is positively autoregulated by the *sae* locus ^{17,73,100}, and also activated by Agr ^{4,15,73,100,104} and SarA ^{15,105} while it can be repressed by SigB and Rot ^{15,17,73,100} as well as high concentration of NaCl and acidic conditions ¹⁵. Other factors can also induce P1 activation: hydrogen peroxide (H₂O₂) ^{15,73,106}, antimicrobial peptides ⁷³, low pH ^{18,73}, subinhibitory concentrations of antibiotics ^{15,24,102} and human neutrophil peptides ¹⁵. Previous studies showed that Sae plays an important role for the pathogenicity of *S.aureus* because it's essential for regulation of virulence-genes expression *in vivo* ^{4,72}: toxins, adhesins, proteins ^{101,107,17,73}. SaeRS can upregulate the transcription of a-, b- and g-haemolysins ^{17,18,105}, fibronectin-binding proteins, coagulase ^{23,24,72,105}, nuclease ^{17,24,72,105}, protease SspA ^{17,24,72}, Spa, Eap, Emp ^{17,72} and DNase I ²⁷. On the other hand, *saeRS* system can have negative effect on the transcription of capsular polysaccharide ^{17,72}.

The ferric uptake repressor (Fur)

Iron is an essential nutrient for most living organism including *S.aureus* ^{65,108}. This nutrient is important for survival and pathogenesis of bacteria by regulating toxins, enzymes and adhesins ¹⁰⁹ inducing biofilm formation ¹¹⁰. It plays a role in various key metabolic processes ¹⁰⁷ and for the growth of the bacteria.

However, iron is not in free state in the blood circulation ¹¹¹. It can be carried by proteins, such as transferrin ^{65,109,112–114}, lactoferrin ^{65,113,114}, ferritin, and iron± sulfur proteins ¹⁰⁹ but most of the iron is contained in erythrocytes ^{111,112,115}.

Therefore, *S.aureus* has developed different mechanisms to take iron such as siderophores that are iron-scavenging molecules (staphyloferrin A and staphyloferrin B) ^{108,109,114} encoded by *sfaABCD* and *sbnABCDEFGHI* ⁶⁸, the aureochelin ^{109,114}, the Isd system (iron-regulated surface determinant system involving *isd* genes *isdA*, *isdB*, *isdCDEF*, *isdG*, *isdH*, and *isdI*) and htsABC (heme transport system)

^{65,111,113,115,116}. In addition, *S.aureus* can also capture iron using hydroxymate siderophores produced by others bacteria ⁶⁸.

All these mechanisms leading the capture of iron are under the dependence of the global regulator Fur (ferric uptake repressor) ^{107,109} which is not just important for iron acquisition ^{107,114,115}, but interferes in oxidative stress ^{107,109,110,115}, protection against neutrophils ^{68,107,115}, avoidance of immune system, biofilm formation ^{107,110,115}, virulence ^{107,115} and survival (Figure 3). Thus, Fur appears to be a central major global regulator for *S. aureus*. It is a homodimeric metalloprotein containing two domains: N-terminal DNA binding domain and C-terminal dimerization domain ⁶⁸. It shares similarity with PerR and Zur ¹¹⁷ by binding to their Fur-box, located in the promoter region ^{68,117} after making a complex with iron ⁶⁸.

This regulator can positively regulate cell surface proteins such as Eap and Emp which play a role in biofilm formation ^{107,110}, immunomodulatory proteins ¹¹⁵ such as formyl peptide receptor-like 1 inhibitory protein (FLIPr), coagulase, superantigen-like exotoxins (Ssl 1, Ssl 2, Ssl 6, Ssl 7, Ssl 9, and Ssl 11) ¹¹⁵, ferritin (FtnA) ¹⁰⁷ and catalase (KatA) ^{68,107,114}.

On the other hand, Fur can also decrease production of hemolysins, cytotoxins, leukotoxin (LukED) ¹¹⁵, the putative iron-siderophore transport ^{109,114} and several proteins including lipase (LipA) ^{107,115}, phospholipase C (Plc), alkyl hydroperoxide reductase (AhpC) ¹¹⁵, iron surface determinants (IsdAB) ^{107,115}. It can repress capsule synthesis genes (*cap5D*, *cap5G*, and *capH*) and the purine ribonucleotide biosynthesis genes *purE*, *K*, *Q*, *C*, and *L* ¹⁰⁷.

It can be autorepressed or repressed by Agr. Fur can activate the expression of Sae, Agr, Rot in low iron condition¹⁰⁷.

The staphylococcal respiratory response SrrAB

The staphylococcal respiratory response (SrrAB) also called SrrSR ⁸⁸ is a two component system in *S.aureus* ¹¹⁸⁻¹²¹. It has been reported that *srrAB* locus sequence contains two ORFs that overlap by 20 nucleotides ^{37,119}: *srrA* encoding a 28-kDa response regulator with 241-amino acid and *srrB* encoding a 66-kDa histidine kinase with 583 amino acids ^{37,119}. SrrB is located in the membrane and can be autophosphorylated leading to the phosphorylation of SrrA localized in the cytoplasm which will induce the modulation of target genes ^{37,118}.

SrrAB was described in the regulation of different genes involved in survival and growth under oxygen-limited conditions ^{120,121}, in the energy metabolism ^{122,123}, in the biofilm formation ^{121,124}, in the fermentation ^{118,121}, in the detoxification, in the repair system ¹²⁰ and in the adhesion.

Thus, SrrAB can repress *spa* ^{37,118}, *hla* ¹¹⁶, *tst* ^{118,119,123}, *icaR* ^{121,124} and *agr* ^{27,118,121,124} transcription leading to the increase or enhancement of biofilm formation ^{121,124} and can activate *ica* expression under anaerobic conditions ¹¹⁸. However, in presence of oxygen, SrrAB induces upregulation of *tst*, *spa* ¹²⁴, and *icaR* by binding their promoters leading to decrease biofilm formation ^{121,124}.

The flavohemoglobin gene *hmp* ^{68,120} involved in the NO₂-sensing transcription repressor (NsrR) ⁶⁸ and Rex involved in homeostasis maintenance ¹²⁰ are also regulated by SrrAB.

SrrAB can positively autoregulate itself ^{37,119}, but this system can be regulated by others regulators such as Rex ¹²⁰ and Agr ¹²² which inhibit its transcription ^{120,122}.

Factors such as anaerobic condition ^{37,120,124}, stress ¹²⁰, pH ³⁷ can activate it ¹²⁴. Menaquinone, intermediate of oxidative respiratory pathway ¹²² can also modulate its transcription.

LytSR

The two-component regulatory system LytSR consists in a sensor histidine kinase (LytS) and a response regulator (LytR) ^{81,125}. LytSR is involved in autolysis ^{51,125–128} and hydrolysis by repression of peptidoglycan hydrolases ^{51,126}. It can also positively regulate *lrgA*, an anticholin molecule ^{47,80} leading to negative control of murein hydrolase (NorG) ^{27,51,81,117,127,129} important for growth, bacterial lysis and cell integrity ¹²⁹. LytSR is also involved in biofilm formation ¹²⁸.

The vancomycin resistance associated sensor/regulator VraRS

VraRS, another two-component sensory regulatory system ¹³⁰ belongs to "cell wall stimulon" ^{130–133} constituted by the response regulator *vraR* and a sensor histidine kinase *vraS* ^{132,134}.

According to Kuroda *et al.* ¹³⁵, it seems to regulate murein monomer precursor synthesis (MurZ) and polymerization of peptidoglycan ^{135,136}. Thus, VraRS has a role in cell-wall biosynthesis ^{130,131,134,135} and in protection against cell damage ^{22,128,136}.

Besides that, it can upregulate proline/glycine-betaine transporters (ProP and OpuD), stress response proteins (PrsA), peptidases (SpsA, CtpA) ¹³⁵. It has also been reported that VraRS is involved in antibiotic resistance by a positive regulation of glycosyltransferase genes giving glycopeptide resistance ^{122–124,126,135,137,130–132,134,135,138} and resistance to beta-lactam ^{128,130,132,134,135}.

Concerning its regulation, some studies showed that exposure to vancomycin activates its expression ^{130,134} as well as other cell-wall synthesis inhibitors ¹³⁰ such as teicoplanine, b-lactams, ceftizoxime, imipenem, bacitracin, D-cycloserine ^{135,136}, nukacin ISK-1 ¹³⁸. VraRS can also be upregulated in low level of penicillin-binding protein (PBP2) and can autoregulate ¹²⁸ itself after fixation of VraR in its own promoter ^{132,136}.

The glycopeptide resistance-associated GraSR

GraSR is another two component system composed by a cytoplasmic domain histidine kinase GraS ^{139,140} which belongs to the 'intramembrane sensing kinase' ¹⁴⁰ and a response regulator GraR ¹⁴¹, member of the OmpR subfamily of response regulators ¹²⁸. Recently, it was shown that GraSR is a multicomponent system which is associated with an ABC transporter (VraFG) and an accessory cytosolic protein called GraX ^{140,142}, which acts by signaling through the GraS kinase ¹⁴⁰. GraS is a component of antibiotic peptide sensor (APS) ¹⁴². GraS induces glycopeptides ^{137–139}, class I bacteriocins, nukacin ISK-1, bacitracin, nisin A ¹³⁸, daptomycin ¹⁴², gentamicin and vancomycin resistance in *S.aureus* ^{133,138,139,142}. It's also involved in other resistance mechanisms ^{22,139,143} such as hyperthermia and oxydative stress ¹²⁸ and resistance to superoxide radicals by positive regulation of the *mntABC* manganese transporter genes ¹²⁸.

GraSR is known to control the response to CAMPs ¹⁴⁰ (major components of host innate immune defense systems) in *S. aureus* ^{128,140–142,144} by regulating two factors: the membrane protein DltB and the lysyltransferase Mpr ^{128,138–140,142,144}. These two proteins influence the cell surface charge of bacteria ¹³⁸. DltABCD is responsible of D-alanylation of cell wall teichoic acid and MprF induces lysinylation of phosphatidyl-glycerol within the cell membrane ^{140,141,144}. These CAMPs include calcium-complexed daptomycin (DAP) (a cationic lipopeptide complex), polymyxin B (PMB) (a prokaryotic cationic cyclopeptide), human neutrophil peptide 1 (hNP-1) ¹⁴² and defensins ^{138,140}. On the other hand, GraSR induces upregulation of VraFG ^{128,133,141,143}, involved in export of components which alter cell surface

properties ¹⁴³. It can also interact with WalkR, a TCS (Two Component System), involved in cell wall metabolism and autolysis ¹²⁸.

However, GraSR expression also can be modulated by VraFG ^{140,144}. *In vitro* experiments reveal that GraSR can also be regulated by CAMPs, such as tPMP-1 (platelets) and polymyxin B ¹⁴⁴.

The nicin susceptibility associated NsaRS

It is another member of TCS family ²², orthologue of GraRS in *S. aureus* ²² with maximal transcription during the exponential and post-exponential growth phases ²². It's a TCS¹³⁸ which includes a response regulator NsaR and a sensor histidine kinase NsaS ¹⁴³. It plays a role in antibiotics resistance such bacitracin ^{22,138} and nisin ^{22,138,143}.

Kolar et al. ²² have also shown that NsaRS is important for biofilm formation, survival, metal ion transport, biosynthesis of surface proteins, cells protection against host immune system and virulence by upregulation of genes (eg *sarS*, *spa*, *srtB*, *clpB* and *isaB*, *V8 protease*, *staphopain B*, *FnpB*, *Hlb*...). It has been shown that it can upregulate 211 genes ²². Phosphomycin, D-cycloserine affecting intracellular peptidoglycan assembly, gramicidin, nisin and CCCP (carbonyl cyanide m-chlorophenylhydrazone) upregulate expression of NsaRS ²².

The multiple-antibiotic-resistance Mar

MarR is the regulator of multiple antibiotic resistance ^{29,90,145} in many bacteria. It's a winged-helix protein ^{29,90,146} which shares similarity to SarR and SarS ²⁹. TcaR, SarZ, MgrA are homologues to MarR ¹⁴⁷.

MarR can affect expression of several genes such as SarA ¹⁴⁶. It's a repressor of MarRAB ¹⁴⁶ and it decreases the susceptibility of the bacteria to multiple antibiotics ^{146,147} such as tetracycline, chloramphenicol, b-lactams, puromycin, nalidixic acid and fluoroquinolones ¹⁴⁶. Proteins regulated by MarR can confer to the bacteria resistance to oxidative stress agents, household disinfectants, organic solvents and pathogenic factors ¹⁴⁷.

Most MarR transcription regulators are repressors ¹⁴⁶ and its regulation is under dependance of sodium salicylate, antibiotics, metal ions, cysteine oxidation or phosphorylation ¹⁴⁷.

The teicoplanin-associated locus regulator (TcaR)

It is another regulator that shares amino acid sequence homology with members of the MarR family ⁸⁹. It can increase teicoplanin ⁸⁹ and reduce methicillin resistance in methicillin-resistant *S. aureus* ^{89,135}. It can also affect positively transcription of regulators SarS and thus *spa* expression ^{89,92}, downregulate *sasF* ⁸⁹ encoding for exoprotein and *ica* operon ^{147,148}. *TcaR* expression can be attenuated by salicylate exposure ⁹⁰.

As Agr, VraSR, GraSR, it can contribute to glycopeptide resistance ¹³⁷ but several antibiotics can inhibit TcaR DNA-binding activity ¹⁴⁷.

WalkR

WalkR, also named YycFG or VicRK or MicAB is another two-component regulatory system, essential for some gram positive bacteria as *S. aureus* due to its role in murein hydrolases activities ¹⁴⁹, autolysis ^{128,149}, cell wall metabolism by expression of autolysins ^{128,149} and regulation of peptidoglycan hydrolase (LytM) ¹⁴⁹. Its expression can be modulated by GraSR ¹²⁸.

Conclusion

Staphylococcus aureus is a clinically important pathogen because it's a commensal bacterium that induces several types of infection. To generate those infections, it has to use several virulence genes that are under regulation of different regulators that interact together. It was shown that the regulation of virulence genes of *S. aureus* is complex because many regulators can regulate one gene. In this review, we see the effect of the most important and the most studied regulators of *S. aureus*.

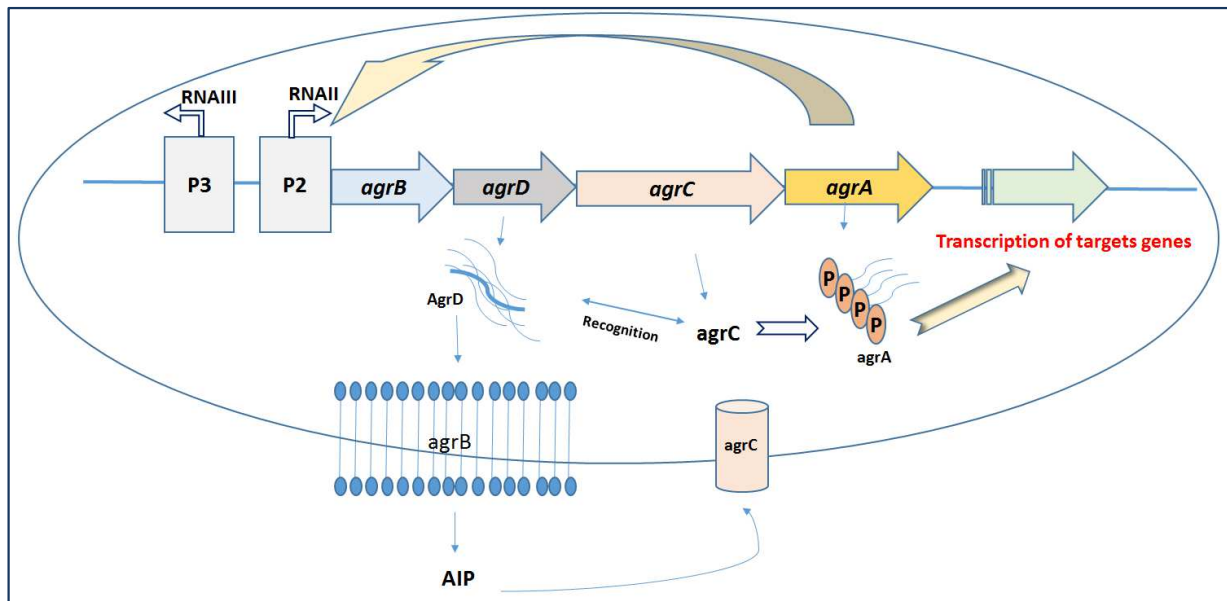


Figure 1: Simplify representation of Agr structure.

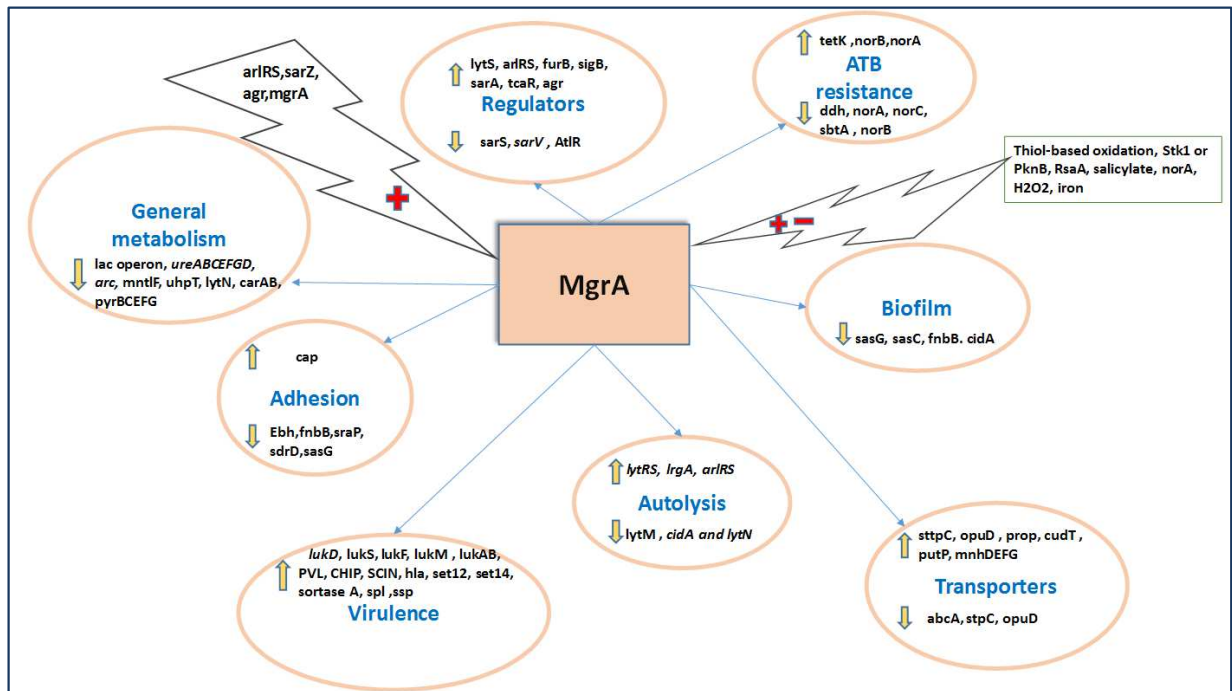


Figure 2: Global regulation of MgrA.

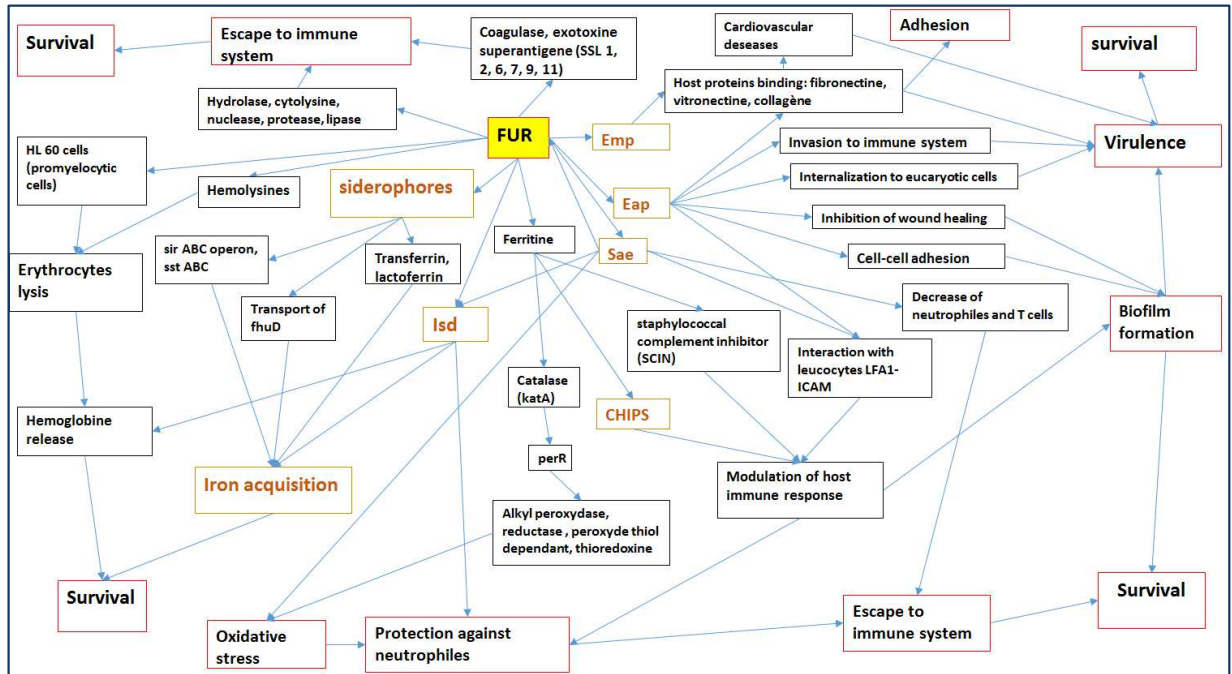


Figure 3: Global regulation of Fur.

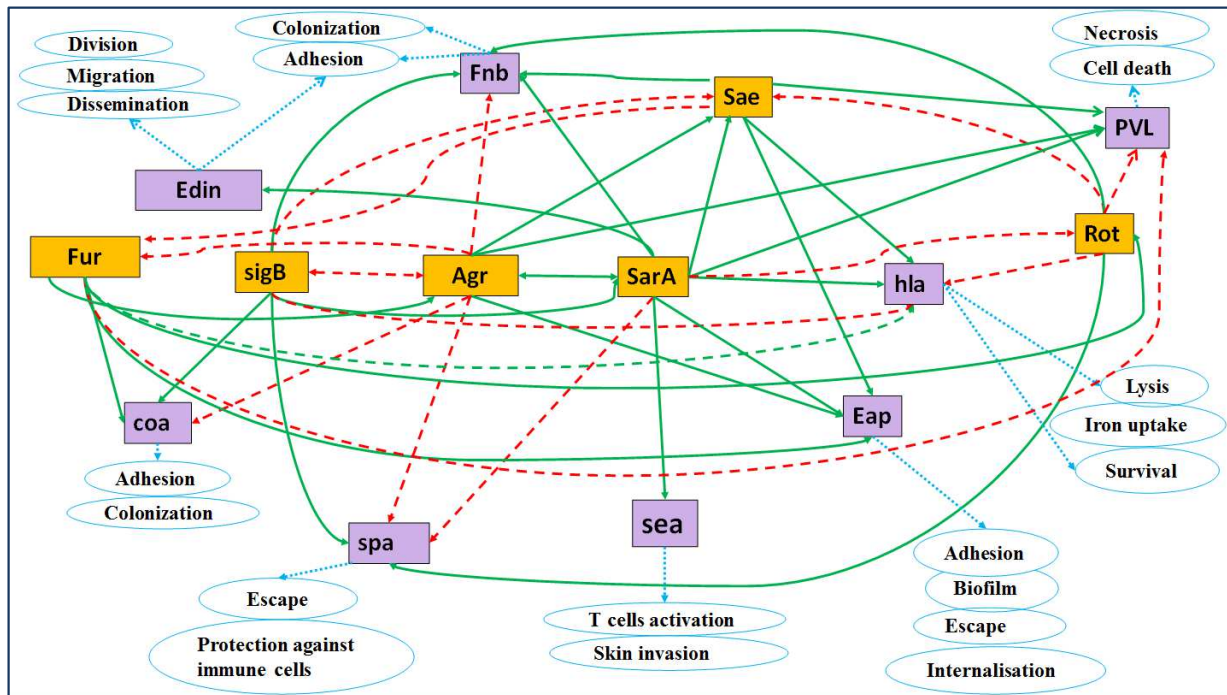


Figure 4: Complex network of virulence genes regulation in *S. aureus*. Solid lines between genes in green represent the upregulation while dotted lines in red color represent downregulation.

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5. Organisation en biofilm

Les bactéries peuvent exister sous forme planctonique (libre) ou sous forme sessile (attachée), formant le **biofilm**. D'après Donlan et Costerton ⁶⁷, le biofilm est une communauté sessile (polymicrobienne) caractérisée par des bactéries qui s'attachent irréversiblement à une surface ou une interface. Ces bactéries sont agglomérées dans une matrice d'EPS (extracellular polymeric substances) qu'elles produisent. Elles présentent un comportement altéré avec des modifications de croissance et d'expression des gènes ⁶⁸. L'attachement de bactéries pionnières est la première étape de la formation du biofilm. A partir de là, les bactéries se multiplient formant des petits agrégats (ou microcolonies). Les autres bactéries colonisantes co-adhèrent à ce biofilm en cours de construction. Cette étape est sous la dépendance de signaux chimiques (auto-inducteurs) permettant la communication bactérie-bactérie essentielle dans la constitution du biofilm. Ces signaux assurent la production d'EPS, les interactions synergiques ou antagonistes entre les bactéries et les changements de phénotype des bactéries. Cette communication entre bactéries est connue sous le nom de *quorum sensing* et donne des informations sur la densité des bactéries. Quand les bactéries ont atteint un seuil critique, elles produisent de l'EPS et certains gènes sont sur/sous-exprimés.

Le biofilm est à l'origine de nombreuses maladies infectieuses comme l'endocardite, la prostatite, la periodontite, l'otite moyenne et de nombreuses infections de matériels médicaux. Potera ⁶⁹ a rapporté que 65% des infections humaines induisaient du biofilm ⁶⁹. Or, les staphylocoques sont responsables d'une grande partie des infections causées par des biofilms ⁷⁰. Une étroite corrélation entre virulence et capacité à former des biofilms a été démontrée chez *S. aureus* ^{70,71}. De multiples facteurs contribuent aux différentes phases de formation du biofilm ⁷⁰:

- 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.

Les conséquences cliniques du biofilm sont importantes. D'abord, les bactéries sessiles sont résistantes aux antibiotiques. La concentration minimale inhibitrice peut être augmentée de 100

à 1000 fois pour des bactéries sessiles par rapport à des bactéries planctoniques ⁷². Plusieurs mécanismes sont à l'origine de cette résistance ^{67,73,74} :

1. la résistance aux antibiotiques : l'EPS peut constituer une barrière physique et/ou chimique empêchant la pénétration des antibiotiques. La modification du micro-environnement peut également altérer l'efficacité des antibiotiques. Par ailleurs, la proximité des bactéries favorise le transfert horizontal des gènes notamment ceux de résistance augmentant donc le nombre de bactéries résistantes au sein du biofilm. Il est également admis que les bactéries sessiles peuvent intervenir dans la régulation des gènes de résistance notamment en augmentant leur expression. D'autres éléments ont encore été identifiés : comme les biofilms sont polymicrobiens, la sensibilité aux antibiotiques est souvent hétérogène ; certaines espèces étant sensibles à l'antibiotique donné et pas les autres. De plus, comme le biofilm est constitué de bactéries dont la croissance est ralentie, l'efficacité des antibiotiques est donc réduite.
2. la modulation de l'expression des facteurs de virulence : Les bactéries sessiles sont plus virulentes que les bactéries planctoniques du fait de la surexpression des gènes de virulence. De plus, comme nous l'avons vu, la proximité des bactéries augmente le transfert horizontal de ces gènes entre bactéries.
3. la résistance des bactéries aux défenses immunitaires de l'hôte : le biofilm provoque une diminution de la pénétration des cellules phagocytaires à travers l'EPS et/ou un défaut d'opsonisation empêchant la phagocytose ^{73,74}.

6. Métabolisme du fer chez *S. aureus*

Un stress environnemental majeur rencontré par les bactéries lors d'une infection est la limitation sévère en fer ³⁸. À l'exception de certains lactobacilles et spirochètes, le fer est un nutriment essentiel pour toutes les bactéries ³⁴. Il est impliqué dans différents processus métaboliques clés, son acquisition est vitale pour la survie bactérienne ³⁸. Le fer joue un rôle essentiel dans les métabolismes cellulaires tels que la respiration et la biosynthèse de l'ADN ⁷⁵. C'est un cofacteur pour de nombreuses autres métalloprotéines ³⁴ et pour les enzymes de défense contre le stress oxydatif telles que la catalase, la peroxydase, et la superoxyde dismutase ⁷⁶. C'est souvent un élément nutritif limitant la croissance bactérienne en raison de sa propension à interagir avec l'oxygène pour former des précipités insolubles ³⁴.

De plus, les niveaux de fer régulent également différents facteurs de virulence tels que les toxines, les enzymes et les adhésines ⁷⁷. *S. aureus* est capable d'utiliser des quantités faibles de fer disponible *in vivo* comme un signal environnemental majeur permettant le déclenchement de plusieurs mécanismes d'acquisition du fer ^{38,78,78}, d'adhérence aux cellules hôtes et de formation de biofilm ^{38,78,79}.

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) ⁸⁰. Il est complexé avec d'autres molécules, telles que les protoporphyrines (hème) ou des protéines (ferritine, transferrine, lactoferrine, hémoglobine...). Pour contourner cet obstacle, *S. aureus* met en place de nombreux systèmes d'acquisition de fer ³⁵. Il s'agit principalement :

- Du **locus *isd*** (Iron-regulated surface determinant) : il catalyse l'extraction de l'hème de l'hémoglobine et le passage de l'hème dans la paroi cellulaire ⁸¹. Il 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 8) ^{43,82}. Ces protéines travaillent de concert afin de permettre au *S. aureus* d'utiliser l'hémoglobine des vertébrés comme source de nutriment fer ³⁵.

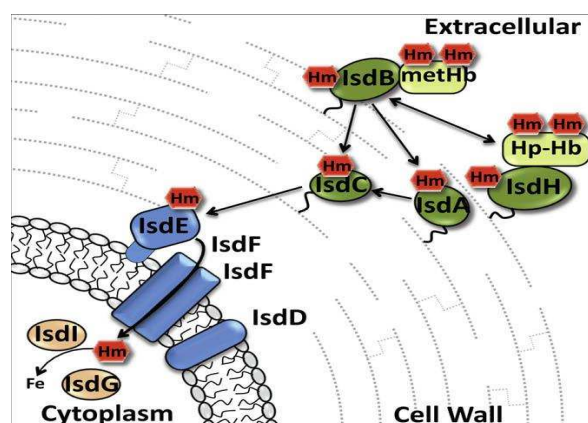


Figure 8 : Représentation schématique des composants du système de transport de l'hème Isd

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- Les **sidérophores** : Ce sont plusieurs petites molécules (en général moins de 1 kDa) ³⁴ qui chélatent le fer de l'hôte et le livrent dans les cellules bactériennes par les systèmes d'absorption

spécifiques ⁷⁷. Trois sidérophores différents (auréocheline, staphyloferrine A et staphyloferrine B) ont déjà été identifiés dans les staphylocoques ⁷⁷. Ces sidérophores fournissent une autre source de fer à *S. aureus* lorsque ceux-ci sont incapables d'acquérir l'hème au cours de l'infection ⁸¹. Ces sidérophores ciblent et éliminent le fer qui est lié à la transferrine et la lactoferrine ³⁵.

Cependant, le fer peut être toxique pour la croissance bactérienne lorsque son niveau dépasse le niveau requis pour satisfaire les besoins physiologiques. En particulier, le fer a la capacité de catalyser la formation de radicaux hydroxyles qui peuvent causer la mort cellulaire. Par conséquent, l'absorption du fer doit être étroitement régulée afin d'éviter les effets toxiques de suraccumulation de fer ⁷⁷. Ainsi, les mécanismes d'acquisition du fer chez *S. aureus* sont sous la dépendance d'un régulateur global, Fur (Ferric uptake repressor) qui intervient non seulement dans la captation de fer mais également dans la régulation des voies métaboliques ⁷⁷, l'expression d'un grand nombre de protéines cytoplasmiques, la formation de biofilm, l'échappement au système immunitaire et affecte l'expression des protéines de stress antioxydatif ^{36,38,79} (cf Figure 3 et article 2 : Complex network of virulence genes regulators in *S. aureus*).

PROBLEMATIQUE GENERALE

Toutes les plaies chroniques étant colonisées par des bactéries, le diagnostic clinique d'infection au niveau d'une plaie du pied chez le patient diabétique est une problématique importante dans la prise en charge de ces infections. Des critères cliniques ont été validés afin de classer l'infection des plaies du pied chez le patient diabétique en séparant les « Grade 1 » correspondant à une colonisation, des « Grades 2 à 4 » correspondant à des infections plus ou moins profondes et sévères. Toutefois excepté les spécialistes dans le domaine de l'infection du pied chez le diabétique, il est difficile de distinguer une colonisation (Grade 1) d'une infection (grade 2) ⁸³⁻⁸⁵. Pour certains experts, il existerait un continuum entre la colonisation et l'infection des plaies chroniques. Les bactéries commenceraient par coloniser le revêtement épidermique cutané puis envahiraient les tissus sous-jacents. Pour passer d'un état à l'autre, les bactéries se multiplieraient et la quantité de bactéries présentes permettrait d'estimer l'état clinique de la plaie ⁸⁶⁻⁸⁸. Une quantité trop importante de bactéries correspondant à une 'colonisation critique' pourrait entraver la cicatrisation de la plaie ou pourrait être le point de départ de l'extension de l'infection. Cependant, l'utilisation de la quantification de la charge bactérienne au niveau des plaies chroniques pour distinguer les infections des colonisations est totalement **inadaptée**. Même en faible quantité (10^3 - 10^4 UFC/g de tissus) identifiée au niveau de la plaie, une bactérie pathogène peut être très virulente pour l'hôte. Au contraire, certaines bactéries commensales peuvent être en grande quantité ($>10^6$ CFU/g de tissus) et n'être que colonisantes. Il est indispensable de tenir compte du **potentiel de virulence** des bactéries et des possibilités de défenses immunitaires de l'hôte. Il est donc évident que les différentes espèces bactériennes isolées de plaies infectées n'ont pas toute la même pathogénicité et plutôt que d'évaluer la quantité de bactéries, il semble préférable : i) de tenir compte des bactéries isolées ; ii) de corréler le potentiel de virulence des bactéries isolées et la présentation clinique.

Or, l'évaluation de ce potentiel de virulence ne se limite pas uniquement au potentiel de virulence d'une espèce donnée car cette virulence est variable au sein d'une même espèce. En effet, des études antérieures menées par notre équipe au CHU de Nîmes ont montré par exemple que toutes les souches de *S. aureus* ne présentaient pas la même virulence. En effet, certaines souches n'étaient isolées qu'au niveau de plaies de grade 1, c'est à dire au niveau de plaies non infectées, ces souches seraient donc peu virulentes. L'étude par biopuces à ADN de ces souches dites 'colonisantes' de *S. aureus* a montré qu'elles appartenaient aux complexes clonaux (CC) CC5/CC8-MSSA (sensible à la méthicilline)^{12,15,16}. D'autre part, lors du suivi prospectif de ces plaies colonisées, plus de 90% des plaies ayant eu une bonne évolution à un

mois présentait des souches de *S. aureus* appartenant toujours aux complexes CC8/CC5-MSSA alors que toutes les plaies ayant eu une aggravation dans le mois ne présentaient pas de *S. aureus* appartenant aux complexes CC8/CC5-MSSA. Ceci nous a permis de montrer que le potentiel de pathogénicité d'une bactérie ne dépend pas uniquement de l'espèce à laquelle elle appartient mais également de son origine clonale et de son évolution au cours du temps (perte/gain d'îlots de pathogénicité, perte/gain de phages ou de plasmides). De plus, la comparaison des génomes des souches infectantes et colonisantes a permis d'identifier, localiser et caractériser une **insertion génétique d'ADN** présentes uniquement chez les souches isolées de plaies chroniques colonisées. Cette insertion permettrait donc de différencier les 2 populations de *S. aureus*. Les comparaisons de séquences dans les banques de données ont permis de déterminer que cette séquence était homologue à celle d'un phage, le **phage ROSA** excepté que cette séquence était inversée par rapport à la séquence connue ⁸⁹. Ce phage a donc été nommé ROSA-like. Des publications antérieures avaient déjà montré l'importance de ces phages dans la modulation de la virulence, mais bien souvent ces phages étaient responsables d'une augmentation du potentiel de virulence ⁹⁰⁻⁹². Ce phage, qui n'est présent que chez des souches de *S. aureus* isolées de plaies de grade 1 appartenant aux CC8/CC5-MSSA serait responsable de la diminution de la virulence des souches colonisantes de *S. aureus* et de l'augmentation de la formation de biofilm. Cependant, des questions demeurent : compte-tenu des conditions environnementales particulières présentes au niveau du pied diabétique, ces conditions de stress pourraient-elles provoquer l'excision du phage et donc restaurer la virulence de cette souche ? D'autre part, nous avons démontré que le phage ROSA-like était inséré dans une région hotspot, proche du locus *isd*, locus intervenant dans l'acquisition du fer chez *S. aureus*. Le fer est indispensable à la survie des bactéries ; comme nous l'avons vu précédemment, il intervient à plusieurs étapes du métabolisme : dans la respiration, la synthèse d'ADN, la formation de biofilm, la toxicité ^{34,38,77}. Compte-tenu du rôle ubiquitaire du fer chez *S. aureus*, quel est donc l'impact de ce phage dans le métabolisme du fer et également dans le métabolisme général de la souche colonisante ?

Au delà des considérations génétiques, d'autres facteurs pourraient influencer la virulence de *S. aureus* au niveau des lésions du pied chez le patient diabétique. En effet, comme nous l'avons abordé précédemment, le pied diabétique est une infection chronique, nécessitant une prise en charge particulière, parfois longue tenant compte des complications souvent associées au diabète sucré telles que l'ischémie et l'artériopathie. En cas d'infection, les bactéries responsables de cette infection vont se développer dans un environnement riche en sucre, souvent peu oxygéné, pouvant être également imprégné d'antibiotiques pendant plusieurs

semaines. Plusieurs études ont été réalisées afin de montrer l'impact de l'hyperglycémie sur l'expression des facteurs de virulence et sur le contrôle de l'infection par *S. aureus*. En 1992, Regassa *et al.* ⁹³ ont montré que la présence de glucose diminuait la production de l'entérotoxine (Sec+) et l'alpha toxine (Hla) à pH 6,5. Cette hyperglycémie avait également une influence sur le système immunitaire et sur le contrôle des infections. En effet, l'hyperglycémie pourrait inhiber des effecteurs du complément C3, élément important dans le contrôle immunologique de *S. aureus* ⁹⁴. En 2014, Mauriello *et al.* ont montré que l'hyperglycémie inhibait le recrutement d'effecteurs humoraux, la production d'anaphylatoxine et l'opsonisation médiée par le complément du *S. aureus* ⁹⁵. D'autres travaux se sont intéressés à l'impact des antibiotiques sur la virulence de *S. aureus*. En 2003, Bernardo *et al.* ont montré qu'en phase exponentielle de croissance, le linézolide inhibait de façon dose dépendante la sécrétion des facteurs de virulence de *S. aureus* incluant les entérotoxines SEA et SEB, la protéine A, l'alpha et la beta hémolysine. En début de phase stationnaire, le linézolide réduisait également la sécrétion des facteurs de virulence et augmentait la sécrétion des exoprotéines comme la triacylglycérol lipase, la glycérol ester hydrolase, le DnaK, ou le facteur d'élongation translationnel EFTu ⁹⁶. D'autres études se sont intéressées à l'impact des antibiotiques sur la production de toxines. Ainsi, Dumitrescu *et al.* ont montré que des concentrations subinhibitrices d'oxacilline augmentaient la production de PVL alors que la clindamycine, le linézolide et l'acide fusidique inhibaient sa production ; la vancomycine n'avait quant à elle aucun effet ⁴⁵. Il a également été montré que la clindamycine, la tigécycline et le linézolide réduisaient la production de PVL et de protéine A. De plus, la clindamycine réduisait l'expression de l'alpha toxine ⁴⁷. La plupart de ces travaux se sont intéressés à l'impact d'une **exposition ponctuelle** à des facteurs environnementaux sans tenir compte qu'au cours de la prise en charge d'une plaie chronique, ces temps d'exposition s'étendent à plusieurs semaines laissant aux bactéries telle que *S. aureus* le temps de s'adapter aux conditions environnementales. Une question peut être posée : Comment évolue la virulence de *S. aureus* après une exposition prolongée à des conditionnements environnementales mimant celles observées au niveau des plaies du pied chez le patient diabétique ?

Parmi les facteurs influençant la virulence de *S. aureus*, les **interactions bactériennes** sont également un élément majeur à prendre en compte. En effet, les infections des plaies aiguës ou chroniques sont souvent polymicrobiennes ⁹⁷. Ces bactéries sont capables de communiquer entre elles en particulier au cours de la formation de biofilm. Les espèces bactériennes associées à *S. aureus* au niveau de ces plaies peuvent être soit des espèces pathogènes telles que *Escherichia coli*, *Proteus mirabilis*, Streptocoques β -hémolytiques soit des bactéries du

microbiote cutané telles que *Staphylococcus epidermidis* et *Corynebacterium* sp. Ces interactions peuvent impacter la virulence de *S. aureus*. En effet, plusieurs interactions entre cette bactérie et d'autres pathogènes ont été décrites : coopération avec *Enterococcus faecalis* et *Candida albicans*^{98,99}, compétition avec *Lactobacillus* sp.¹⁰⁰ et *Streptococcus pneumoniae*¹⁰¹ ainsi que les deux phénomènes combinées (compétition/coopération) avec *Pseudomonas aeruginosa*^{102–105}. D'autre part, des données cliniques ont permis de mettre en évidence une association bactérienne très peu décrite auparavant mais notée très fréquemment à l'heure actuelle grâce à l'avènement de la spectrométrie de masse au niveau des plaies du pied diabétique. Il s'agit de l'association de *S. aureus* avec une bactérie commensale *Helcococcus kunzii*. Cette bactérie est un cocci à Gram positif, catalase-négative, anaérobie facultative et décrit comme pathogène opportuniste^{106–109}. Le rôle de cette bactérie étant actuellement inconnu dans les infections polymicrobiennes, quelle est la virulence des souches cliniques d'*H. kunzii* isolée du pied diabétique et quel est l'impact de cette espèce commensale sur la virulence de *S. aureus* puisque ces bactéries sont très fréquemment associées au cours des infections du pied diabétique ?

Les objectifs de cette Thèse étaient :

- d'étudier la stabilité du phage ROSA-like et l'impact de ce phage dans le métabolisme général et en particulier dans le métabolisme du fer d'une souche colonisante de *S. aureus*,
- d'étudier l'effet prolongé des conditions de stress rencontrées dans le pied diabétique sur la virulence des souches de *S. aureus*,
- d'étudier l'interaction entre *S. aureus* et *H. kunzii* et l'impact de cette interaction sur l'évolution de la virulence de *S. aureus*.

RESULTATS

Travail n°3

Behavior of colonizing *Staphylococcus aureus* strain isolated from diabetic foot ulcers

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Article original : Article soumis à publication « Virulence »

OBJECTIF : Etudier l'impact du phage Rosa-like sur le métabolisme global d'une souche colonisante isolée des plaies de grade 1 de patients diabétiques.

RESUME :

Récemment, nous avons pu isoler des plaies du pied chez le diabétique une souche colonisante de *S. aureus*. La particularité de cette souche est la présence d'un phage nommé ROSA-like, responsable de la faible virulence de cette souche et de l'augmentation de la formation de biofilm ⁸⁹.

1/ Cette souche n'étant détectée que sur les plaies de Grade 1 contrairement aux autres souches infectantes sans phage isolées de plaies de Grade 2-4, nous nous sommes interrogés sur la stabilité de ce phage dans les souches colonisantes.

Utilisant un modèle *in vitro*, que nous avons développé, mimant les conditions environnementales pouvant être présentes au niveau du pied diabétique, nous avons cultivé la souche colonisante sous différentes conditions : forte teneur en sucre (glucose, malose, saccharose), concentrations subinhibitrices d'antibiotiques (linézolide, vancomycine, cloxacilline), anérobiose, hyperthermie, et milieu minimum. La présence ou l'absence du phage a été testée chaque semaine par PCR.

2/ D'autre part, le phage ROSA-like étant inséré dans une région « hotspot » pour l'insertion des phages chez *S. aureus* mais située à côté du locus *isd*, locus intervenant dans l'acquisition du fer chez la bactérie, notre attention s'est portée sur le rôle de ce phage dans le métabolisme du fer de la souche colonisante.

L'impact du phage dans le métabolisme du fer a ainsi été évalué par : l'établissement de courbes de croissance, l'évaluation de la production des sidérophores par le test au chromazurol, la quantification relative de l'expression des gènes *isdA,B,D* et *fur*, les analyses transcriptomiques de l'ensemble de l'expression des gènes présents dans la souche par microarray et des tests d'adhésion sur les cellules de keratinocytes humains HaCaT.

Nos résultats ont montré que le phage était extrêmement stable au sein de la souche colonisante et ne pouvait être excisé quelles que soient les conditions environnementales que nous avons testées.

Par ailleurs, les courbes de croissance dans un milieu pauvre en fer ont montré une absence de croissance de la souche colonisante contrairement aux autres souches de *S. aureus* ne

possédant pas le phage (NSA739) ou possédant un phage différent (Souche de référence Newman). Nous avons également remarqué que dans ces conditions, contrairement aux souches infectantes et contrôles testées dans notre étude qui surexprimaient les gènes du locus *isd*, produisaient des sidérophores et réprimaient le régulateur global *fur*, la souche colonisante continuait à surexprimer le gène *fur* bloquant la production des autres gènes du locus *isd* ; les sidérophores étant eux aussi non produits. Enfin, les études par microarray en milieu appauvri en fer nous ont révélé une baisse d'expression des gènes d'adhésion MSCRAMMs dans la souche colonisante ce qui a été confirmé par les tests d'adhésion qui ont montré un faible pourcentage d'adhésion sur les kératinocytes de cette souche. Les gènes du chimiotactisme CHIPS, les gènes du métabolisme des pyrimidines, la coagulase, les gamma hémolysines étaient également réprimés dans la souche colonisante. Cependant, l'alpha hémolysine, les transporteurs de nickel, les gènes du métabolisme des lipides et du fructose étaient, au contraire, suexprimés dans cette souche par rapport à la souche Newman de référence.

Tous ces résultats suggèrent que dans la souche colonisante, le phage est stable et induit une dysrégulation du régulateur global de fer, *fur*. Ceci entraîne donc un blocage des mécanismes de captation de fer chez cette bactérie, une régulation négative de certaines voies métaboliques intervenant dans la croissance, l'adhésion et la virulence. Néanmoins, cette souche arrive à se maintenir en vie ou survie en stimulant d'autres voies métaboliques (notamment via les lipides et le fructose).

Behavior of a colonizing *Staphylococcus aureus* strain isolated from diabetic foot ulcers

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Key word: *Staphylococcus aureus*, Rosa-like phage, metabolism, iron, fur, commensalism.

Abstract

Recently we discovered a colonizing *Staphylococcus aureus* strain with a low virulence isolated in diabetic foot ulcers (DFU). The main particularity of this strain was the presence of the Rosa-like phage. This study investigates the influence of the phage on the strain metabolism. We evaluated the stability of the phage by using an *in vitro* model mimicking the conditions encountered in DFU, the impact of the phage in the strain metabolism by using a transcriptomic approach and the adhesion on an *ex vivo* HaCaT cells model. Results showed that the ROSA-like phage was stable in the colonizing strain and cannot be excised in stress conditions. Transcriptomic analysis revealed that the phage derepressed the activity of the transcriptional regulator Fur and significantly reduced the growth of the strain and blocked iron uptake. The mutation of the *fur* gene showed that the phage affects the bacteria in a Fur-independent manner. In a low iron environment, the colonizing behavior was explained by a downregulation of the MSCRAMM genes. The decrease of adhesion was confirmed on HaCaT cells.

This study showed that the ROSA-like phage modulates the relationship of *S. aureus* in the human host and promotes chronic colonisation. Such discovery is an important advance in commensalism understanding.

Introduction

Staphylococcus aureus is a Gram-positive bacterium of clinical importance. This opportunistic pathogen is capable of causing a wide range of diseases from skin and soft tissue infections to life-threatening bacteremia, endocarditis, and osteomyelitis.¹ 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. Understanding the mechanisms that drive the transition of *S. aureus* from commensality to invasiveness is important to predict infection risk in colonized patients and to propose targeted decolonization procedures to high-risk patients. Although the molecular epidemiology of carriage and clinical strains can be markedly different,² no genomic characteristic able to firmly discriminate commensal and invasive isolates has been detected so far.³

The transition from colonization to invasion is of particular importance in diabetic foot ulcer (DFU), a frequent and chronic complication of diabetes mellitus. *S. aureus* often colonizes DFU wounds without triggering infection, but it can also invade the underlying tissues, frequently causing diabetic foot osteomyelitis.⁴ We recently published that two markedly different populations of *S. aureus* isolates are present on DFU and that strains of the clonal complex (CC) 5/8 were overrepresented in uninfected ulcers.^{5,6} Genome-wide comparisons of invasive and uninfected strains within CC5/8 led to the identification of a 44 kb phage insertion which was highly specific of uninfected isolates.⁷ The presence of the phage, named ROSA-like, significantly reduces the virulence of the strain and increases the biofilm formation.⁷ This phage is inserted in a hotspot region Φ NM2 near the locus *isd*, the main iron surface determinant that transports heme-iron across the bacterial wall and membrane. Strikingly, the spontaneous expulsion of ROSA-like, selected under iron-depleted growth conditions, restored virulence in the same models.⁷

In this study, we report the impact of this phage in the iron uptake metabolism and the behavior of this strain in iron depleted conditions.

Results

Stability of Rosa-like phage

During previous study, we observed that the excision of the ROSA-like phage restored the bacterial virulence and the potential of invasiveness.^{7,8} To test the stability of this phage, we developed an *in vitro* model that mimics the conditions encountered in DFU. Whatever the conditions used we were not able to detect the deletion of the phage after 24 weeks suggesting the high stability of the Rosa-like phage in colonizing strains. The only exception is the condition using the iron-depleted medium which induces the excision of the phage (NSA1385(P-)) as previously noted.⁷

The genetic island deregulates the iron acquisition

As we observed an abnormal behaviour of the colonizing strain in iron depleted medium condition and as we noted that the phage was inserted near the locus *isd*, we sought to study the impact of the phage insertion on the iron metabolism. We analyzed the behaviour of five strains: the two clinical strains (NSA1385 and NSA739), the reference strain Newman (which harbors a phage insertion at Φ NM2 hotspot), a knock-out mutant of the *isdD* gene (NSA*isdD*::erm), the complemented strain of the *isdD* gene LUG1900 (IsdDNSA*isdD*::erm) and the control strain LUG1899 (pNSA*isdD*::erm). These three strains allowed to evaluate the impact of a mutation in locus *isd* (Table 1).

Concerning the growth curves, we observed that no difference could be detected in LB medium between each strain and a slight reduction of growth of NSA1385 strain in LB supplemented with iron (100 mM) (data not shown). However, a significant difference was noted in LB medium with Chelex 3% at 24h and

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

The expression of the *isd* genes of the strains cultured in LB medium with Chelex 3% highlighted that the colonizing NSA1385 strain didn't increase the expression of the *isdA*, *isdB*, *isdD* genes in this low iron environment (Figure 2). In all the control strains and the infecting NSA739 strain, the expression of these genes was significantly increased compared to the results obtained with the NSA1385 strain ($p < 0.001$). To understand this modification of gene expression, we studied the expression of *fur* gene, the global regulator of the iron metabolism. Interestingly, contrary to the control strains and the infecting NSA739 strain, which presented a repression of this gene in the low iron condition, the colonizing NSA1385 strain presented a high expression of *fur* gene ($p < 0.001$) explaining the absence of the *isd* genes expression (Figure 2). These results suggested that the colonizing NSA1385 strain presented a deregulation of the *fur* gene. Interestingly the Newman strain (which harbours a phage insertion in the Φ NM2) presented behaviour similar to the infecting strains in a low iron condition with a repression of *fur* gene and an overexpression of *isd* genes (Figure 2). This result suggests that the phage ROSA-like could be the cause of the deregulation of *fur* gene.

To determine the impact of this deregulation of *fur* gene, we evaluated the siderophores formation, the second way to acquire iron in low iron medium. Using a chromazurol test, no variation of the OD₆₀₀ (optical density measured at 600 nm) was observed in NSA1385 strain cultured in a LB medium with Chelex 3% after 60 min and a slight decrease was observed after 90 min ($p =$ not significant) (Figure 1B). In contrast, the other studied strains presented a significant decrease of the OD₆₀₀ after 30, 60 and 90 min in a LB medium with Chelex 3% ($p < 0.001$). This result suggests that the colonizing NSA1385 strain had no siderophore formation contributing to the inability of this strain to growth in a low iron condition.

The excision of ROSA-like phage restores the iron metabolism of S. aureus

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 phage expulsion (Table 1). We observed the restoration of bacterial growth in low iron environment associated with the derepression of the *fur* gene and the increased expression of the *isd* genes (Figures 1A and 2). Moreover, we noted the restoration of siderophore production (Figures 1B). These data suggest that the phage ROSA-like clearly influences the virulence of the colonizing *S. aureus* strain notably disrupting its iron metabolism by a deregulation 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 determined if the Fur protein is active in addition to being transcribed in iron depleted conditions. Fur is a protein that also uses iron as a cofactor. This means that the Fur protein becomes inactive in low iron environments. It could therefore be necessary to determine whether this protein was active in its stringent condition. In this purpose, we evaluated the transcription of two genes positively regulated by Fur in iron-restricted conditions but not involved in the *S. aureus* iron uptake system: *xerD* (encoding a tyrosine recombinase) which is located in a same operon as *fur* and *sbi* (encoding a *S. aureus* binder of IgG). We observed that both genes were induced in low iron environment in the colonizing NSA1385 strain while these genes were repressed in the infecting strains (NSA739, Newman) and in the NSA1385(P-) (Figure 2). These results suggest that the transcription of *xerD* and *sbi* genes is correlated with the transcription of *fur* gene in NSA1385 strain demonstrating that the Fur protein was active in iron-restricted conditions.

In the aim 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*fur::tet*) strain and a *fur* knock-out mutant of the NSA1385(P-) (1385(P-)*fur::tet*) (Table 1). Firstly, we evaluated the metabolism of iron assimilation from these two strains by following their growth in a restricted iron environment. The 1385*fur::tet* strain was unable to grow in iron-limited environments while 1385(P-)*fur::tet* showed a growth kinetic partially affected (Figure 1A). Secondly, we evaluated the transcription of the *isd* locus, *xerD* and *sbi* genes and the production of siderophores. These two pathways are still affected in the mutant 1385*fur::tet* even with the deletion of *fur* gene (Figure 2). However the expression of *xerD* and *sbi* genes was repressed confirming the absence of the Fur production. The 1385(P-)*fur::tet* strain restored partially the iron assimilation (Figures 1A and 2). We could conclude that the genetic insertion affects the virulence and the iron assimilation system in a Fur-independent manner.

Rosa-like phage affects the metabolism of colonizing S. aureus strain

To understand the role of Rosa-like phage in the global metabolism of the colonizing *S. aureus* isolate in iron restricted conditions, we performed a transcriptomic analysis by comparing the behaviour of the NSA1385 and the reference strain Newman. Our results indicated that in LB + Chelex 3% medium, 36.5% of genes were overexpressed, 28.7% underexpressed and 34.8% of genes had the same expression level. We used qRT-PCR to validate the relative expression levels of selected genes, including several genes found to be differentially regulated by microarray analysis.

Several metabolic pathways have been affected differently in low iron medium. We selected to represent the genes that were significantly differently expressed in NSA1385 compared to Newman strain with an average fold change of at least 3 (Table 2). Globally, in iron-depleted condition, a consistent upregulation of genes implicate in arginine, proline and urea metabolism (*argC*, *arcBCD*), fructose metabolism (*fruAB*), lipid metabolism (*fadE*), nickel/cobalt transporter (*opp-1BCDF*) could be noted. While genes implicated in pyrimidine, alanine, aspartate and glutamate metabolism (*pyrBCRP*, *carAB*), nitrogen metabolism (*narGHK*, *nasD*) are downregulated. Those results suggest that NSA1385 use fructose, arginine, lipid and nickel pathway for growth and maintenance in iron depleted conditions (Figure 3).

Concerning the virulence genes, an upregulation of capsules genes (*capAB*) and some defence virulence genes (*geh*, *norA*, *spxA*) could be observed. However most of the virulence genes are significantly downregulated: MSCRAMM/adhesion genes with -6 to -25 fold change (*empbp*, *efb*, *fmbA*), leucotoxins with -8 to -14 fold change (*hlgABC*), coagulase (*coa*), chemotaxis (*chp*).

To confirm the poor potential of adhesion to host cells, flow cytometry-based adhesion assays were performed using human keratinocytes (HaCaT cell line). Our results show that NSA1385 has a significantly lower adhesion efficiency compared to NSA739 (Figure 3). Not only the % of infected cells is lesser (~3% for NSA1385 *vs* ~13% for NSA739), but the fluorescence intensity of infected cells is also lower with NSA1385, showing that fewer bacteria are attached to the infected keratinocytes with this strain.

Discussion

S. aureus is one of the most prevalent pathogen isolated from DFU.⁹ Recently, we described the existence of a colonizing *S. aureus* strain isolated from this wound.⁷ The particularity of this strain is the presence of a genetic determinant (ROSA-like phage) associated with a low pathogenicity of the bacteria.⁷ The results demonstrated that the prophage was associated with the ability of the bacteria to colonise chronic ulcers and was responsible for the non-invasive character.

In our previous study we observed that the phage excision induced the restoration of bacterial virulence. Some authors have shown that certain factors (e.g. physical stress, chemical stress, ultraviolet light, reactive oxygen species) can provoke phage excision.¹⁰ Here we evaluated different stressful conditions encountered in DFU. We confirm the difficulties to induce phage excision, demonstrated a great stability of

this insertion suggesting a low ability to spread or transfer to other staphylococci.

The importance of iron acquisition in the *S. aureus* virulence was well established and the central role played by Fur was previously demonstrated.^{11,12} Iron plays an essential role in the pathogenicity of *S. aureus*. It is involved in many cellular processes including DNA synthesis, respiration, and defence against reactive oxygen and nitrogen species.¹³ During the infection process, and after passing the epithelial barrier, the bacteria might find itself in a state of stress since over 90% of the iron is sequestered inside the cells and are not available for the bacteria.^{14,15} To overcome this condition, *S. aureus* triggers the expression of iron acquisition systems by the down-regulation of the major regulator Fur. *S. aureus* has two main ways for iron acquisition: (i) the direct acquisition by Isd proteins that absorb iron in the form of heme. This is the main way to the infectious process as suggested by the studies showing that the protein surface IsdB is essential for *S. aureus* virulence;¹⁵ (ii) the indirect acquisition by siderophores which allow the acquisition of free iron in the medium or the iron complexed to transferrin.¹⁴ Fur is a transcriptional repressor that prevents the transcription of genes involved in iron acquisition by binding to a consensus sequence called the Fur box. This repression begins when *S. aureus* are grown under iron availability conditions.^{16,17} In an iron-restricted environment, the expression of *fur* is de-repressed resulting in the expression of the two iron acquisition systems.

We showed that the presence of the phage ROSA-like affected the ability to assimilate iron by the bacteria due to a de-repression of the Fur protein. We observed that the continuous expression of Fur was responsible for the decline of the virulence of the colonizing NSA1385 *S. aureus* affecting the iron uptake machinery in contrast to the phage contained by Newman strain. We noted that the deletion of the *fur* in the NSA1385 strain did not restore bacterial virulence. However, no lifting of repression was observed in the 1385*fur::tet* strain suggesting that the inhibition of all the genes regulated by Fur is due to the ROSA-like phage. We could conclude that the de-repression of Fur is independent and concomitant to the deregulation of the genes regulated by Fur. These results can be explained by the expression of a transcription factor homologous to Fur or by the expression of a putative miRNAs encoded by the ROSA-like sequence. Studies will be needed to identify the factor responsible for the deregulation of the iron acquisition machinery such as RNA sequencing or random mutagenesis.

Interestingly the colonizing strain harbors classical virulence factors (e.g. capsule, enterotoxin A, hemolysins, leukocidin DE, some MSCRAMM factors, and iron-acquisition proteins). MSCRAMM factors, fibrinogen, fibronectin were not produced by this strain, explaining its non-adherence in human keratinocytes, the first step to establish pathogenicity before penetrating host tissues and produce toxins.^{18,19} Furthermore, pyrimidine (important for DNA synthesis, reparation and for bacterial replication²⁰) and nitrogen metabolisms (use for ATP biosynthesis) were inhibited. This strain produced also less coagulase or γ -hemolysin, explaining its ability of the bacteria to colonize wound and to live in a commensal state.

Despite the non-replication of colonizing strain in iron depleted medium, we observed its maintenance. In complex medium, *S. aureus* primarily consumes glucose, arginine, and threonine.²¹ Transcriptomic analysis revealed that the colonizing *S. aureus* strain uses fructose and arginine pathway, fatty acid and urea cycle that lead to activate glycolysis and glycogenesis pathway ending to the energy production. This strain also stimulates ABC transporter Opp that helps bacteria to the capture of other metal (cobalt and nickel) in the aim to allow its survival in absence of iron.^{22,23}

The presence and the stability of ROSA-like phage explain the ability of the bacteria to colonize chronic wound tissues and their ability to exist in the commensal state. This phage represents the first description of a mobile element present in *S. aureus* strains, which modulates the relationship of the bacteria with its human host by attenuating bacterial virulence and could be an important progress in our understanding of commensalism.

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), LB + 100µM of iron, and LB + Chelex-100 (Sigma) resin (3% w/V). 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 described previously.¹⁶ Briefly, sequences flanking the *isdD* coding sequence were cloned into pOS1 and interrupted with an *ermC* cassette. Inactivation of *isdD* was achieved through allelic replacement of the native *isdD* locus with these vectors associated sequences.

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 3).

Introduction of the *fur* mutation into the colonizing NSA1385 strain was achieved by phage transduction, performed as previously described,²⁴ from *S. aureus* 8325-4 *fur* mutant²⁵ using Φ11 as the transducing phage.

Evaluation of spontaneous phage excision

In the aim to detect and evaluate spontaneous excision of ROSA-like phage from the hotspot region ΦNM2, we used an *in vitro* DFU-like model adapted from DeLeon et al. that mimics the conditions encountered in this wound.²⁶ The model consists to cultivate the colonizing NSA1385 strains in a wound-like medium, grown under static conditions at 37°C and successively inoculated over 24 weeks in environments with sugars 2-10% (glucose, saccharose, maltose), minimum media, anaerobic conditions, hyperthermia (42°C) and in presence of a sub-inhibitory antibiotic concentrations (0.5x MIC) cloxacillin, linezolid and vancomycin.

To detect phage insertion/deletion we used a PCR protocol previously described.⁷

Real Time RT-PCR

Analysis of the mRNA (transcription) levels of the *isdA*, *isdB*, *isdD*, *fur* genes was performed following the method previously described by Doumith et al.²⁷ Briefly, total cellular RNA was extracted using the Rneasy® Mini kit (Qiagen, Courtaboeuf, France) and treated with RNase-free DNase (Qiagen) for 30 min at 37°C, after which a second step of purification was performed. RT-PCR was carried out in a LightCycler® using the one-step LightCycler® RNA Master SYBR Green I kit (Roche Applied Science, Meylan, France) according to the manufacturer's protocol. The specificity of the generated products was tested by melting-point analysis. 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 were listed in Table 3.

Siderophores production

An assay solution similar to that described by Schwyn and Neilands²⁸ was prepared 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 bay adding 100 mg of 5-Sulfosalicylic acid. The CAS Shuttle solution was stored in the dark.²⁸

The presence of siderophore will result in a reduction in blue color which can be measured at an absorbance of A_{630} 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 A_{630} 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}) : $A_{630-S}/A_{630-R} = \% \text{ siderophore units}$.²⁸

Microarray analysis

The microarray was manufactured by the *in situ* synthesis of 10807 60-mer long oligonucleotide probes (Agilent, Palo Alto, CA, USA), selected as previously described.²⁹ It covers 98% of all ORFs annotated in different reference strains (N315, Mu50, MW2, COL, NCTC 8325, USA300, MRSA252 and MSSA476 (including their respective plasmids)).³⁰⁻³²

Total RNA was purified from exponential phase bacteria growth in LB broth or in LB with 3% of chelex and treated with DNase. Preparations of 5 μg of total *S. aureus* RNA obtained after grown in LB/chelex broth were labeled with Cy-3 dCTP and RNA obtained after growth in LB broth were labeled with Cy-5 dCTP using the SuperScript II method (Invitrogen, France) and purified, as previously described.³³ Mixtures of Cy5-labelled DNA and Cy3-labelled cDNA were hybridized and scanned, as previously described.³³ Hybridization fluorescence intensities were quantified using Feature Extraction software (Agilent, version 8). Data from two independent biological experiments were analysed using GeneSpring 8.0 (Silicon Genetics, Redwood City, CA, USA), as previously described,³³ with a 5% false discovery rate (P value cut-off, 0.05) and an arbitrary threshold of 1.5-fold for defining significant differences in expression ratios. Microarray results were confirmed by qRT-PCR in the same conditions described previously.

Adhesion assay

Human keratinocytes (HaCaT cell line) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Twenty-four hours before the adhesion assay, cells were detached by trypsinization and the concentration of live cells was determined after propidium iodide (PI) staining (1 $\mu\text{g}/\text{mL}$, 10 min), using the absolute counting function of a MACSQuant flow cytometer (Miltenyi Biotec). PI positive cells were considered as dead or altered and were excluded from the counts. Cells were seeded at a density of 2×10^4 cells/well in 96-wells culture plates (Falcon) and incubated at 37°C in a CO₂ incubator.

Bacteria were fluorescently labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA/SE, Molecular Probes) as previously described³⁴ and applied to HaCaT cells with a multiplicity of infection (MOI) of 500. After 1h30 of incubation, cells were washed twice with PBS to remove non-adherent bacteria, gently detached by trypsinization (25 μL Trypsin-EDTA), resuspended in 100 μL of medium containing 10% FBS, homogenised and transferred into a 96-well V-shaped plate (Greiner). Cells were immediately analysed by flow cytometry (MACSQuant, Miltenyi Biotec) in the B1 channel (488 nm laser). Quantification was measured using the MACSQuantify software (Miltenyi Biotec). The efficiency of bacterial interaction with HaCaT cells was expressed either as the % of fluorescent cells or as the MFI (Median Fluorescence Intensity) of fluorescent cells.

Statistical analysis

For each qualitative variable, comparison was assessed using the Fisher's exact test. The Chi square-test was used to compare the mean values of the normalized genes transcription. The Kruskal-Wallis test was used to compare the *in vitro* bacterial growth of the different strains.

Acknowledgments

Fer-15 nematodes were provided by the *Caenorhabditis* Genetics Center, a foundation of the NIH National Center for Research Resources (NCRR). We thank René Courcol for his fruitful comment, and Mariella Lomma for her editing assistance.

Disclosure of potential conflicts of interest

No potential conflict of interest was disclosed.

Funding

This work was supported by grants from the French Society of Diabetes (ALFEDIAM grant 2008), the Languedoc-Roussillon Region (Chercheur d'avenir award 2009) and the National Institute of Health and Medical Research.

C.N.E. and N.M. are PhD students from the Foundation Mediterranean Infection.

TKP was supported by a British Infection Association (BIA) research fellowship.

EPS was supported by a grant from the National Institutes of Health (R01 AI069233).

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Figure Legends

Figure 1 Growth curves and Siderophore production for the *S. aureus* strains.

A) Growth curves of the different *S. aureus* strains studied in presence of LB + chelex 3%.

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

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

Figure 2 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.

Figure 3. Adhesion efficiency of NSA1385, NSA739 or Newman to human keratinocytes.

A) Percentage of cells infected by NSA1385, NSA739 or Newman. Non-infected cells were used as a negative control.

B) Median fluorescence intensity (MFI) of cells infected by NSA1385, NSA739 or Newman.

Mean values and standard deviations are shown and were calculated from three independent experiments.

Table 1 Main characteristics of the *S. aureus* strains and plasmids used in the study

Strains/Plasmids	Characteristics	References
Newman	reference strain	-
NSA739	<i>S. aureus</i> strain isolated from infected diabetic foot ulcer (Grade 3)	6
NSA18026	<i>S. aureus</i> strain isolated from infected diabetic foot ulcer (Grade 4)	6
NSA1322	<i>S. aureus</i> strain isolated from colonized diabetic foot ulcer (Grade 1)	6
NSA1385	<i>S. aureus</i> strain isolated from colonized diabetic foot ulcer (Grade 1)	6
NSA1385 (P-)	<i>S. aureus</i> strain isolated from colonized diabetic foot ulcer (Grade 1) after expulsion of the phage	7
NSA <i>isdD</i> ::erm [®]	<i>S. aureus</i> Newmann strain with a deletion of <i>isdD</i> gene	This study
LUG1899	pNSA <i>isdD</i> ::erm, ●●●●●●●● NSA <i>isdD</i> ::erm with the plasmid pLUG985 used for complementation	This study
LUG1900	IsdDNSA <i>isdD</i> ::erm, strain NSA <i>isdD</i> ::erm with the complementation of <i>isdD</i> gene (pLUG988)	This study
1385 <i>fur</i> ::tet	NSA1385 strain with a deletion of <i>fur</i> gene	This study
1385(P-) <i>fur</i> ::tet	NSA1385(P-) strain with a deletion of <i>fur</i> gene	This study
pLUG985	pSK265::PlukS(EcoRY)(BamHI), Cm ^R	This study
pLUG988	pSK265::PlukS(EcoRY) <i>isdD</i> (BamHI), Cm ^R	This study

Table 2 *S. aureus* NSA1385 genes differentially expressed by microarray in LB chelex 3% medium compared to Newman strain

Locus	Description	Genes	Function	Fold change NSA1385 vs Newman
Upregulated genes				
Amino acid and nucleotid metabolism				
SAOUHSC_00149	N-acetyl-gamma-glutamyl-phosphate reductase	<i>argC</i>	Arginine and proline metabolism	3.25
SAOUHSC_01129	carbamate kinase	<i>arcC</i>	Arginine and proline metabolism;purine metabolism;nitrogen metabolism	3.75
SAOUHSC_02967	arginine/oimithine antiporter	<i>arcD</i>	Arginine transport	3.57
SAOUHSC_01128	ornithine carbamoyltransferase	<i>arcB1</i>	Arginine and proline metabolism	3.81
SAOUHSC_00434	transcription activator of glutamate synthase operon	<i>gluC</i>	Glutamate metabolism	5.18
SAOUHSC_01435	thymidylate synthase	<i>thyA</i>	pyrimidine metabolism	6.82
SAOUHSC_02289	threonine dehydratase	<i>ilvA</i>	Glycine metabolism/ serine and threonine metabolism/ Valine, leucine and isoleucine biosynthesis	4.29
Carbohydrate metabolism				
SAOUHSC_00707	fructose 1-phosphate kinase	<i>fruB</i>	Fructose and mannose metabolism	3.61
SAOUHSC_00708	fructose specific permease	<i>fruA</i>	Fructose and mannose; Glycolysis / Gluconeogenesis;Galactose metabolism;	3.82

			Amino sugar and nucleotide sugar metabolism	
Fatty acid metabolism				
SAOUHSC_00198	putative acyl-CoA synthetase	<i>fadE</i>		6.86
Lipid metabolism				
Metabolism of Cofactors and Vitamin				
SAOUHSC_02381	general stress protein 20U	<i>dps</i>	Porphyrin and chlorophyll metabolism	4.21
Transporteur				
SAOUHSC_02763	oligopeptide transporter ATPase subunit	<i>opp-1F</i>	Nickel/cobalt transport	3.37
SAOUHSC_02764	oligopeptide transporter ATPase subunit	<i>opp-1D</i>	Nickel/cobalt transport	4.53
SAOUHSC_02765	oligopeptide transporter permease	<i>opp-1C</i>	Nickel/cobalt transport	4.80
SAOUHSC_02766	oligopeptide transporter permease	<i>opp1B</i>	Nickel/cobalt transport	5.19
SAOUHSC_00691	undecaprenyl pyrophosphate phosphatase	<i>bacA</i>	Glycan Biosynthesis and Metabolism (Peptidoglycan biosynthesis)	3.93



Regulators			
SAOUHSC_02799	staphylococcal accessory regulator T, putative	<i>sar</i>	Target genes involving virulence, autolysis, biofilm formation, antibiotic resistance, oxidative stresses, and metabolic processes. 11.06
Virulence capsule			
SAOUHSC_03000	capsular polysaccharide biosynthesis protein capA	<i>capA</i>	Adhesion/biofilm 4.42
SAOUHSC_02999	capsular polysaccharide biosynthesis protein capB	<i>capB</i>	Adhesion/biofilm 3.64
Toxins			
SAOUHSC_02240	truncated beta-hemplysin	<i>hlyb</i>	Cells lysis 5.06
SAOUHSC_01121	alpha-hemolysin precursor	<i>hlyA</i>	Cells lysis 4.29
Other virulence and defense genes			
SAOUHSC_00934	transcriptional regulator Spx	<i>spxA</i>	Stress regulator; function in controlling the expression of oxidative stress response genes 3.50
SAOUHSC_02239	integrase	<i>int</i>	Integration, recombination of phage 6.79
SAOUHSC_00300	glycerol ester hydrolase	<i>geh</i>	Invasion and destruction of host tissue 34.79
Antibiotic resistance			



SAOUHSC_00703	quinolone resistance protein	<i>norA</i>	efflux pump, antibiotic resistance gene	3.29
Downregulated genes				
Amino acid and nucleotid metabolism				
SAOUHSC_01400	alanine racemase	<i>alr</i>	D-Alanine metabolism	-4.96
SAOUHSC_01164	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase	<i>pyrR</i>	pyrimidine metabolism	-3.47
SAOUHSC_01165	uracil permease	<i>pyrP</i>	pyrimidine metabolism	-6.85
SAOUHSC_01166	aspartate carbamoyltransferase catalytic subunit	<i>pyrB</i>	Pyrimidine metabolism; Alanine, aspartate and glutamate metabolism	-5.44
SAOUHSC_01168	dihydroorotase	<i>pyrC</i>	Pyrimidine metabolism	-3.60
SAOUHSC_01169	carbamoyl phosphate synthase small subunit	<i>carA</i>	Pyrimidine metabolism; Alanine, aspartate and glutamate metabolism	-3.07
SAOUHSC_01170	carbamoyl phosphate synthase large subunit	<i>carB</i>	Pyrimidine metabolism; Alanine, aspartate and glutamate metabolism	-3.72
Energy Metabolism				
SAOUHSC_02680	nitrate reductase beta chain narH	<i>narH</i>	Nitrogen metabolism	-3.45
SAOUHSC_02681	respiratory nitrate reductase alpha chain	<i>narG</i>	Nitrogen metabolism	-4.55

SAOUHSC_02684	nitrite reductase	<i>nasD</i>	Nitrogen metabolism	-3.93
SAOUHSC_02671	nitrite extrusion protein	<i>narK</i>	Nitrate/nitrite transporter	-3.62
Metabolism of Cofactors and Vitamin				
SAOUHSC_00722	para-aminobenzoate synthase, glutamine amidotransferase, component II	<i>pabA</i>	Folate biosynthesis	-3.86
MSCRAMM/adhesion				
SAOUHSC_00816	secretory extracellular matrix and plasma binding protein	<i>empbp</i>	cell surface proteins, biofilm formation, adhesion	-25.22
SAOUHSC_01114	fibrinogen-binding protein	<i>efb</i>	MSCRAMM/adhesion	-6.45
SAOUHSC_02803	fibronectin-binding protein A	<i>fibA</i>	MSCRAMM/adhesion	-6.49
Toxins				
SAOUHSC_02708	gamma-hemolysin chain II precursor	<i>hlgA</i>	Toxins, cells lysis	-16.27
SAOUHSC_02709	gamma-hemolysin component C	<i>hlgC</i>	Toxins, cells lysis	-14.44
SAOUHSC_02710	gamma-hemolysin component B	<i>hlgB</i>	Toxins, cells lysis	-8.01
Other virulence and defense genes				
SAOUHSC_00192	staphylocoagulase precursor	<i>coa</i>	Biofilm, immune evasion, fibrinogen-	-6.37

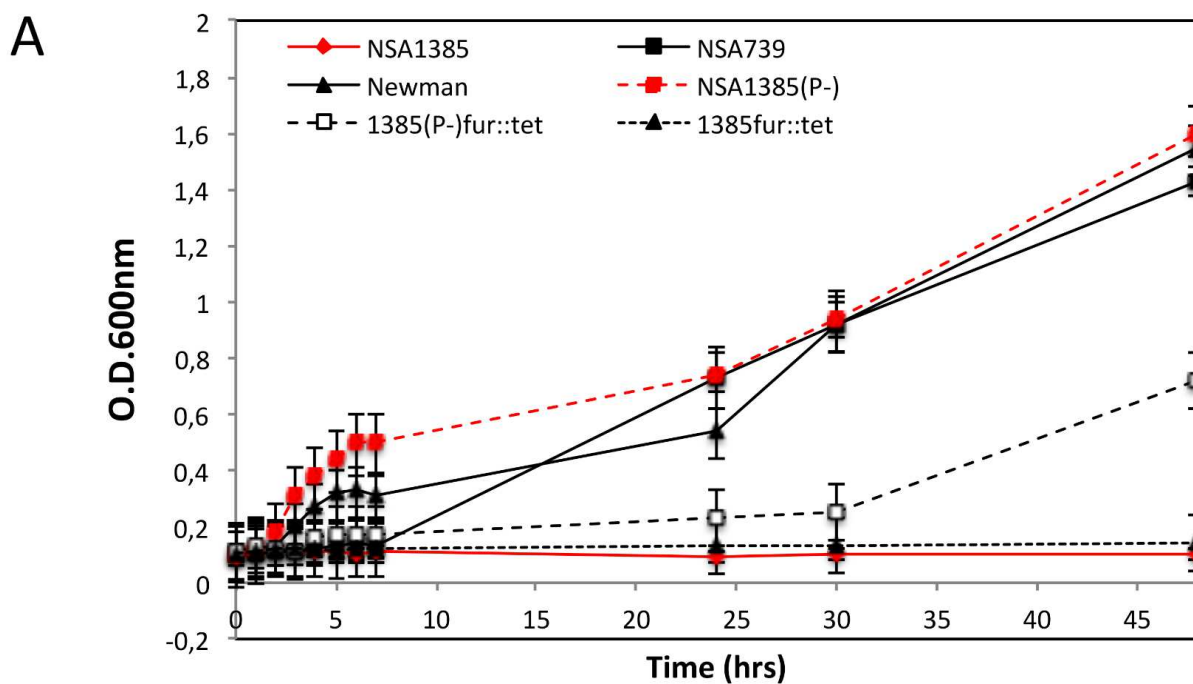
SAOUHSC_02169	chemotaxis-inhibiting protein CHIPS	<i>chp</i>	binding activity, procoagulant Inhibition of neutrophils recruitment	-12.20
SAOUHSC_02160	map protein, programmed frameshift	<i>map</i>	<i>S. aureus</i> antigen; extracellular protein; affects T-cells functions and interferes with neutrophils recruitment	-3.76



Table 3 Primers used in the study.

Name	Primers	Size
Rpm-F	5'-TGGCAGTACCAAAAAGAAGAACTTC-3'	428 bp
Orf34-R	5'-CGGCTGGGAATTAATGGAAG-3'	
Isd-R	5'-GACACACTCAAAGCCAAAACAATAA-3'	
Orf28-F	5'-GATACTCTTCAAAATGAAATGAGCG-3'	
GyrB-F	5'-AGTAACGGATAACGGACGTGGTA-3'	150 bp
GyrB-R	5'-CCAACACCATGTAAACCACCAGAT-3'	
IsdA-F	5'-AAGATAATGAACCTAAAAGGGC-3'	
IsdA-R	5'-GCCTAAACAAGCATCTAAAGTAAA-3'	
IsdB-F	5'-TGTCATCTTTTGGCTGTCTTG-3'	139 bp
IsdB-R	5'-GCCAATACCGATAAATCTAACA-3'	
IsdD-F	5'-GCATGTAAAACGTTGGCTG-3'	
IsdD-R	5'-GGCACTTCTTTTGCATCATC-3'	
Fur-F	5'-GGTTGGCGACAGTATACAGC-3'	288 bp
Fur-R	5'-ACATTCCATACATACTAAATGATG-3'	
XerD-F	5'-AAGGACTAAGTTCTAACACAATTGG-3'	
XerD-R	5'-CTTCGTCAACATTTAAAACG-3'	
Sbi-F	5'-AATATATCTCGAAGTTGCTAGTTGG-3'	469 bp
Sbi-R	5'-GCTTTATCCGCATTTTCAAT-3'	
Isd4836*	5'-GT <u>GATATC</u> CATGAGAAATGTTAAACAAATTGC-3'	
Isd5929*	5'-TTA <u>GGATCCT</u> CAAACCTTGTTACC-3'	

Figure 1 Growth curves and Siderophore production for the *S. aureus* strains. (A) Growth curves of the different *S. aureus* strains studied in presence of LB + chelex 3%. (B) Siderophore formation for the studied strains after 30, 60 and 90 min evaluated by chromazurol formation. Bars represent the standard deviations from three independent experiments with triplicate for each one.



B

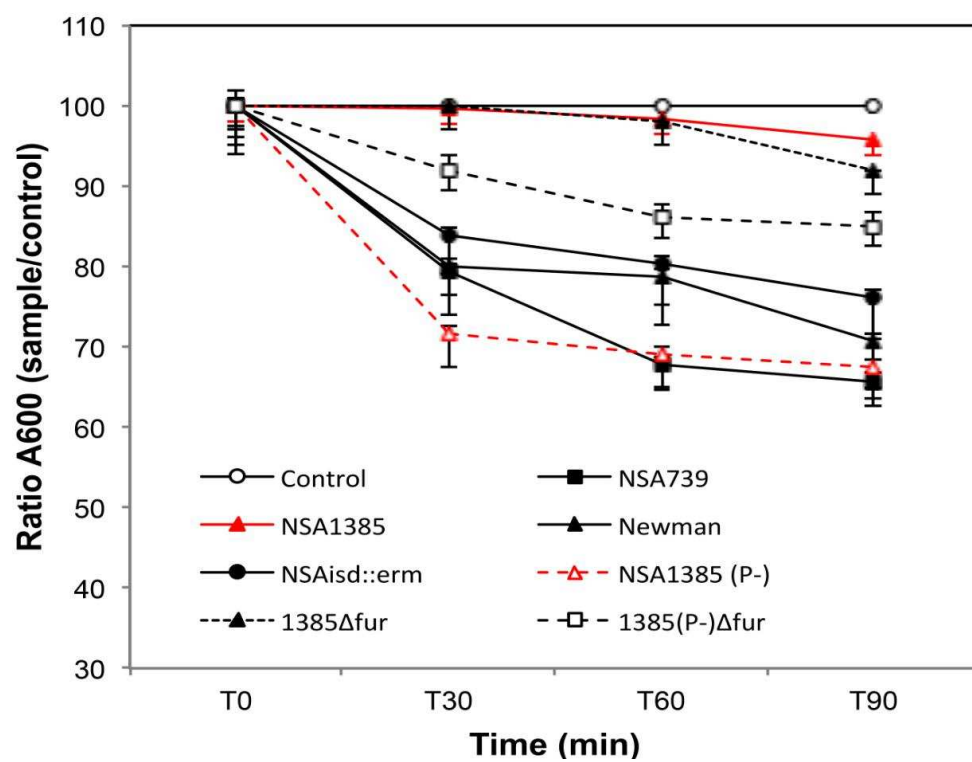


Figure 2 Relative mRNA expression levels of different genes in low-iron conditions. Mean and SD of genes expression relatively to that of strains in LB medium are indicated.

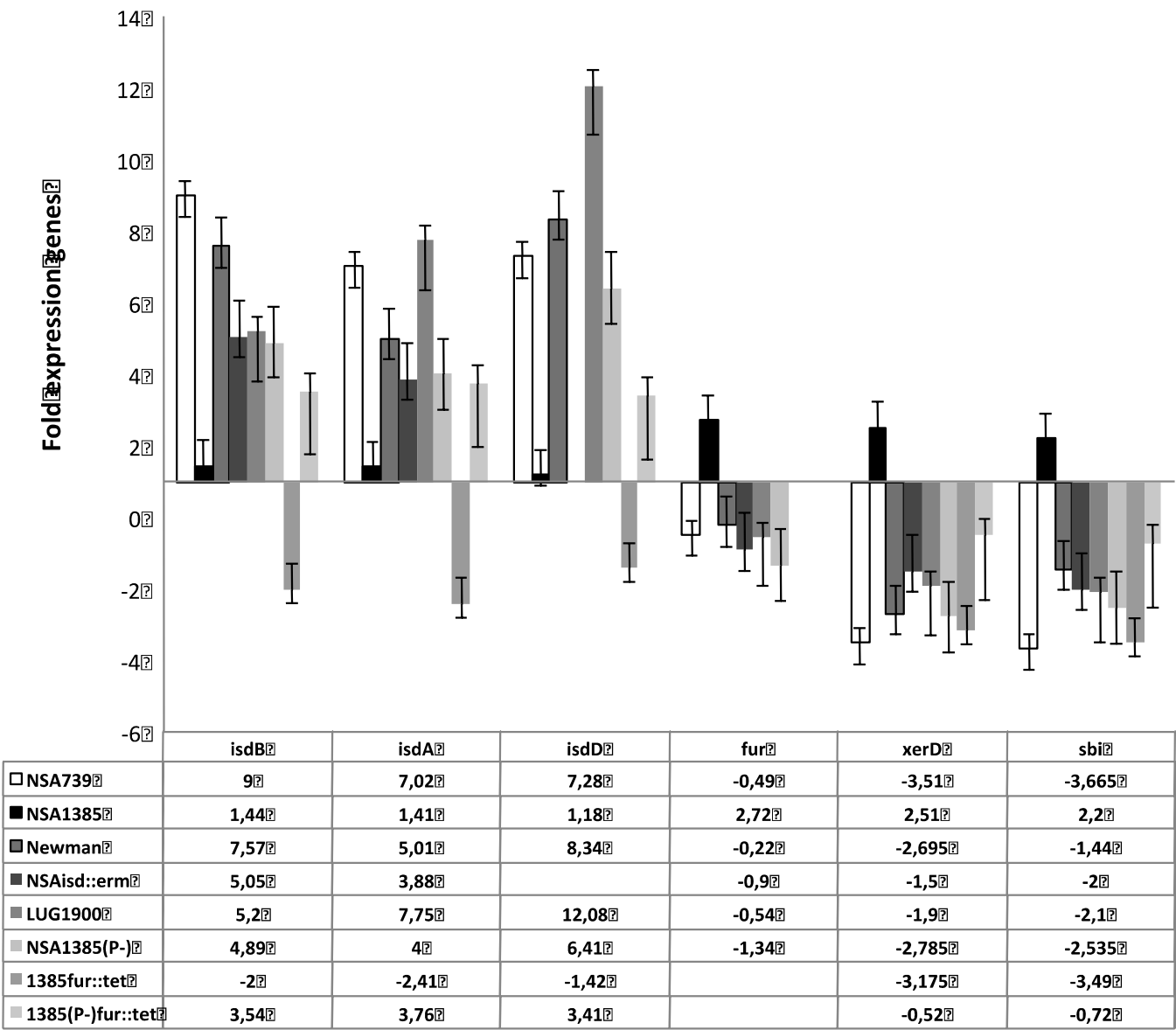
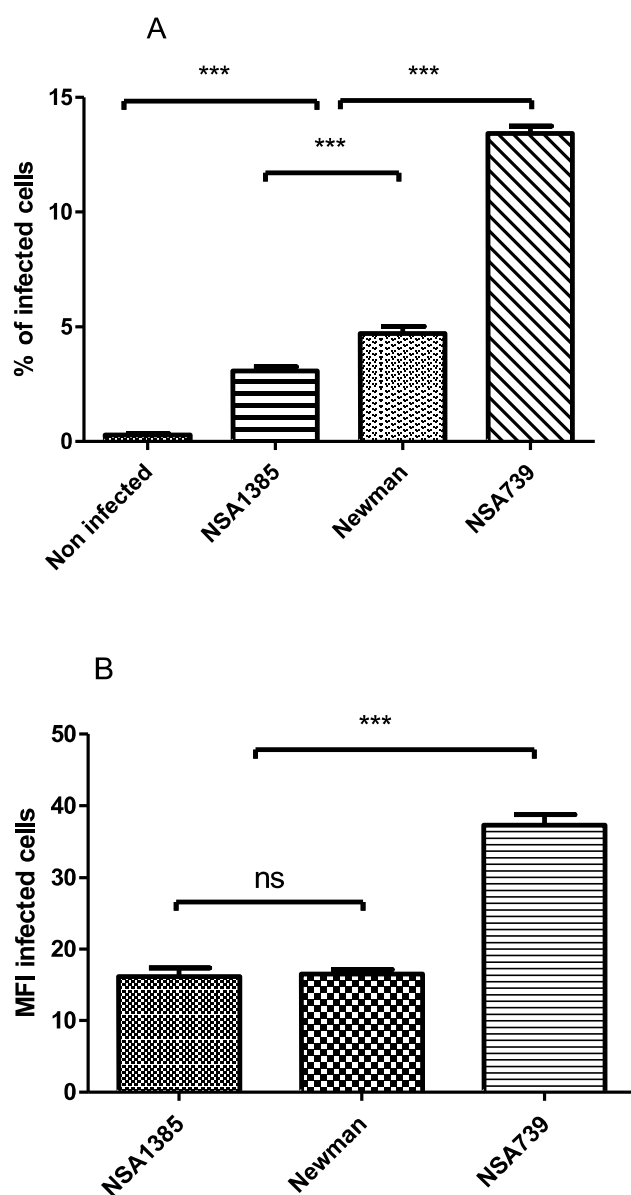


Figure 3 Flow cytometry analysis of cells infected with NSA1385, NSA739 and Newman.

The mean values and standard deviation of median fluorescence intensity (MFI) and the percentage of infected cells calculated from three independent experiments are indicated. A. Comparison of percentage of infected cells by NS1385, 739, Newman. Non infected cells were used as control. B. Comparison of MFI of the different infected cells.



Travail n°4

***In vitro* model to study adaptation of *Staphylococcus aureus* in prolonged environmental stress conditions**

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Article original : Article en préparation

OBJECTIF : Etudier l'effet prolongé de différentes conditions de stress rencontrées dans le pied diabétique (présence d'antibiotiques, anaérobiose, forte teneur en sucre) sur la virulence des souches de *S. aureus*.

RESUME :

Le diabète sucré est caractérisé par une élévation en glucose dans le sang entraînant de nombreuses complications telles que l'ischémie, l'artériopathie, la neuropathie à l'origine de l'ulcération du pied diabétique. Ces plaies s'infectant très fréquemment (dans plus de la moitié des cas) les antibiotiques sont fréquemment prescrits dans cette population de patients. Tout cela contribue à faire du pied diabétique un environnement particulier nous poussant à nous interroger sur l'impact de ces conditions de stress à court terme et à long terme sur le potentiel de pathogénicité de *S. aureus*, principale bactérie responsable des plaies du pied chez les diabétiques.

Pour répondre à ces questions, nous avons développé un modèle *in vitro* nous permettant de mimer les conditions environnementales rencontrées dans le pied diabétique. Ce modèle consistait à cultiver pendant 16 et 24 semaines différentes souches cliniques représentatives des souches de *S. aureus* isolées de plaies du pied chez le diabétique dans différentes conditions environnementales : hyperglycémie, concentrations subinhibitrices d'antibiotiques (linézolide et vancomycine), anaérobiose. L'expression des gènes codant pour les principaux facteurs de virulence du *S. aureus* (*lukF/lukS-PV*, *edinB*, *edinC*, *hla*, *spa*, *eap*, *fnb*) ainsi que le régulateur global (*agr*) a été évaluée par qRT-PCR. De plus, des études de virulence sur le modèle *C. elegans*, des études phénotypiques et des expériences de biofilm ont également été réalisées.

Après 16 et 24 semaines de culture, l'apparition de small colony variants (SCVs) a été notée. Ces petites colonies sont métaboliquement inactives et n'induisent pas de réponse immunitaire. Ce changement phénotypique étant caractéristique du *S. aureus* en situation d'infection chronique, cela nous a permis de valider notre modèle comme étant un modèle de chronicité. Les différentes souches ont été ensuite testées sur notre modèle de virulence *C. elegans*. Une baisse significative de la virulence des souches de *S. aureus* étudiées a été notée. Les études transcriptomiques ont confirmé une baisse d'expression des gènes de virulence majeurs de *S. aureus* : *edinB*, *edinC* et *lukF/lukS-PV* après une culture prolongée dans le glucose, les antibiotiques et en anaérobiose. De même une baisse d'expression des gènes codant pour l'hémolysine bêta dans toutes les conditions a été notée. Ces résultats ont été confirmés par la diminution de formation de biofilm dans le glucose et la vancomycine et la baisse de virulence sur le modèle *C. elegans*. A l'opposé, de façon intéressante, les études transcriptomiques ont montré une activation de certains gènes de virulence (*hla*, *eap*, *fnb*).

Dans cette étude, nous avons donc pu développer un modèle d'étude d'infection chronique mimant les conditions de stress rencontrées dans le pied diabétique. L'étude de l'adaptation du *S. aureus* après plusieurs semaines nous a montré globalement une baisse de la virulence des souches de *S. aureus* dans une forte teneur en glucose, en anaérobiose et en présence de concentrations sub-inhibitrices d'antibiotiques. Cette étude nous a également permis de montrer le rôle important des antibiotiques principalement du linézolide dans la lutte contre *S. aureus*.

***In vitro* model to study adaptation of *Staphylococcus aureus* in prolonged environmental stress conditions**

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Keywords: *Staphylococcus aureus*, sugar, antibiotic, stress, expression, adaptation, chronic wound, adaptation.

Abstract

Objectives: *Staphylococcus aureus* (SA) is the most prevalent pathogen isolated in diabetic foot ulcers (DFU). Very few publications have focused on the virulence and the adaptation of this pathogen in chronic wound conditions. The purpose of this study was to develop an *in vitro* model miming the conditions encountered in DFU in the aim to determine the adaptation of SA in these environments.

Methodology: The *in vitro* model consisted to cultivate during 24 weeks representative clinical SA strains by successive inoculations in environments with: glucose 10%, antibiotics at 0,75x MICs (linezolid, vancomycin), anaerobia, associations between sugars and antibiotics. The expression of the main virulence (*luk-F-pvl*, *edinB*, *edinC*, *hla*, *spa*, *eap*, *fnb*) and the global regulator (*agr*) genes was evaluated by qRT-PCR. Phenotypical test, biofilm and *C. elegans* experiments were performed.

Results: In the conditions miming the DFU environments, we observed the apparition of small colony variants (SCVs) and the loss of beta-hemolysin production. Long exposure to excess of glucose, antibiotics (linezolid and vancomycin) and anaerobia leads the downregulation of main virulence genes of *S. aureus* Edin and PVL and reduction of biofilm formation. However *S. aureus* tries to survive by activation of other virulence factors such as *hemolysin*, *spa*, *eap*.

Discussion: We have developed a new promising *in vitro* model miming the conditions encountered in DFU. This model allowed evaluating the adaptation of SA to prolonged stress environmental conditions. All these results allow a best comprehension of the SA virulence in chronic wound conditions and confirm the important role of utilization of antibiotics to SA infections.

Introduction

Diabetes mellitus is a public health problem representing the third cause of mortality and morbidity in the world ¹. One of the main complications of diabetes are foot ulceration due to ischemia, neuropathy, and arteriopathy²⁻⁴. Infection in diabetic foot ulcer (DFU) is particularly frequent and represents one of the major cause of lower-limb amputation ⁵.

DFU are polymicrobial and *Staphylococcus aureus* is the most frequent pathogen isolated ⁶⁻⁸. This Gram-positive coccus is a leading cause of a wide range of diseases from skin and soft tissue infections (e.g. impetigo, carbuncles) to life-threatening bacteraemia, toxic shock syndrome, endocarditis, and osteomyelitis ⁹. This bacterium, also commensal organism notably in nasal cavity, is a real pathogen in DFU. The comprehension of the *S. aureus* pathogenicity in this disease remains still unknown.

Staphylococcus aureus deploys an arsenal of virulence factors to colonize, invade and destroy host immune cells and tissues ¹⁰. Those virulence factors are adhesins that bind to different host proteins MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) as fibronectin binding proteins (FnBPs) and extracellular matrix binding proteins (Eap) which are important for adhesion and colonization ^{9,11,12}. Eap is also known to be important for inhibition of wound healing, thus it plays a role in chronic infections ^{12,13}. After adhesion, *S. aureus* uses toxins and exoproteins to destroy host tissues and disseminate as alpha-hemolysin (Hla) and Panton–Valentine Leucocidin (PVL) that are pore-forming toxins ¹⁴⁻¹⁶, exoenzymes EDINs (EDIN-A, -B and -C), which inactivate the small GTPase RhoA, to promote the dissemination of the bacteria ^{17,18}, and protein A (Spa) to escape to immune system and protect against host-mediated clearance ¹⁹. In addition, other virulence factors are secreted by *S. aureus* to inactivate lymphocyte T cells as the superantigens (SE: enterotoxins). All these virulence factors are dependent of different regulation factors (such as Agr).

During diabetic foot infection (DFI), *S. aureus* is exposed at different stress conditions as elevated glucose concentration, the decrease of tissue oxygenation, and also the presence of antibiotics during several weeks. Some studies analyzed the impact of antibiotics and glucose in some *S. aureus* strains ^{16,20-22} during a punctual exposition, but very few data have focused on the virulence and the adaptation of this pathogen after exposition at these conditions on a long time period, reproducing the chronic wound conditions.

The purpose of this study was to develop an *in vitro* model miming the conditions encountered in DFU in the aim to see the impact of those specific conditions on the virulence of *S. aureus* in acute and chronic infection and to determine the adaptation of *S. aureus*.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1.

NSA739, 1077 and 7475 are infecting strains isolated from deep DFI (diabetic foot infection). The strain 1077 was PVL+ and EdinB+; the strain 7475 was EdinC+. NSA739 was also isolated from DFI, NSA1385 was a colonizing strain isolated from uninfected ulcer and the reference *S. aureus* strain Newman was used as control. The avirulent *Escherichia coli* OP50 was used as a food source and a control for the nematode assays.

We used an *in vitro* DFU-like model adapted from DeLeon *et al.* that mimic the conditions encountered in the wound ²³. The strains were grown in different conditions described in Figure 1. Thus, bacteria were grown at 37°C with shaking in Luria Bertani (LB) broth supplemented with 10% of glucose and/or sub-inhibitory concentration of antibiotics (sub-MICs, 0.75xMIC linezolid and vancomycin). Anaerobic bags (AN0010, AnaeroGen Compact Oxoid) were used to create anaerobic

condition. Every day, the optical density was adjusted at OD 0.1. These cultures were maintained during 16 to 24 weeks (Figure 1).

The vancomycin and linezolid MIC (Minimum Inhibitory Concentration) were determined for each strains by microdilution method in MH (Muller Hinton) medium as recommended by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) ²⁴.

100 µl of Culture on Columbia agar supplemented with 5% fresh sheep blood (bioMérieux, Marcy l'Etoile, France) at 37°C were performed each week to control the loss of beta hemolysin in each condition.

Nematode Killing Assay

The *C. elegans* infection assay was carried out as previously described using the Fer-15 mutant line, which has a temperature sensitive fertility defect.¹² Fer-15 was provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). To synchronize the growth of nematodes, eggs were collected using the hypochlorite method. Overnight cultures of *E. coli* strains in nematode growth medium (NGM) were harvested, centrifuged, washed once and suspended in phosphate buffered saline solution (PBS) at pH 7.0 at a concentration of 10⁵ CFU/mL. NGM agar plates were inoculated with 10 µL of bacterial suspension and incubated at 37°C for 8–10 h. Plates were brought back to room temperature and seeded with L4 stage *C. elegans* (≈30 per plate). Plates were then incubated at 25°C and scored each day for live nematode under a stereomicroscope (Leica MS5). A nematode was considered dead when it no longer responded to touch. *C. elegans* that died as a result of having been trapped by the wall of the plate were excluded from the analysis.

At least three replicates repeated twice were performed for each selected strain. Lethal time 50% (LT50) corresponded to time (in days) required to kill 50% of the nematodes. Three replicates were performed for each condition.

Feeding behaviour assays

For occupancy assays, each bacterial strain was cultured overnight at 25°C in LB media, spotted as a circular lawn on NGM plates and dried at room temperature for 20 min. Approximately 30 L4 animals were carefully placed in the centre of the bacteria lawn. The number of nematodes inside or outside each lawn was counted after 16h as previously described.⁵⁰ The results were explained in percent occupancy corresponding to the number of *C. elegans* in the bacterial lawn/the total number of nematodes. The experiments were performed in triplicate.

The number of bacteria within the *C. elegans* digestive tract was obtained as previously described.⁵¹ Briefly, nematodes were picked at 72 h, and the surface bacteria were removed by washing the nematodes twice in M9 medium containing 25 µg/ml gentamicin. The *C. elegans* were then mechanically disrupted in M9 medium containing 1% Triton X-100.¹² Serial dilutions of the sample were then plated on LB-agar and the colonies were counted after 24 h. Three replicates were performed for each strain.

qRT-PCR assays

Transcriptomic analysis was performed to quantify virulence genes expression of *S. aureus* in different stress conditions after 24h, 16 and 24 weeks. The virulence and regulator genes tested were adhesins (*fnbA*, *eap*), toxins (*hla*, *lukS/luF-PV*, *sea*), exoenzymes and exoproteins (*edinB*, *edinC*, *spa*) and the global

regulator *agr*.

Briefly, overnight *S. aureus* cultures obtained in different media were diluted at an OD₆₀₀ of 0,1 in MH broth and these diluted cultures were cultured again until an OD₆₀₀ of 0.5-0.8. Total RNA from bacteria pellet was extracted as described by the manufacturer using RNeasy Mini kit (Qiagen, Courtaboeuf, France). RNA was treated with the RNase-Free DNase Set (Qiagen, Courtaboeuf, France). Purity and concentration were determined using the NanoDrop™ 2000 spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA). cDNA was synthesized from 1 µg of total RNA for each sample, using the iScript™ Select cDNA Synthesis Kit (Bio-Rad) with random primers according to the manufacturer's instructions.

Real-time PCR assays were performed in a LightCycler®480 device using the LightCycler FastStart DNA Master^{PLUS} SYBRGreen I kit (Roche, Meylan, France) with 100 ng of cDNA and 10 pmol of target primers (Table 2). The specificity of the PCR products was tested by melting point analysis. Amplifications were performed in triplicate from three different RNA preparations. The $2^{-\Delta\Delta CT}$ method was used to analyze transcriptional changes in target genes using *gyrB* and *HU* as the housekeeping control genes (Table 2). Data were log-transformed to obtain a fold change difference between samples and data analysis was performed with the Pfaffl method ²⁵. Error bars indicate the standard deviation (SD) of three independent experiments.

Biofilm formation

To evaluate the biofilm formation, we used the Biofilm ring Test® (BioFilm Control®, Saint Beauzire, France) following the manufacturer recommendations ²⁶. Briefly, this assay is based on the immobilisation (or not) of magnetic beads embedded by bacterial aggregates during biofilm formation. It is realized on modified polystyrene 96-wells microtiter plates with individual 8-wells slides. The image analysis applied on the BioFilm Ring Test® with the software BFCE3 gives a value of the biofilm formation going to 0 (100% biofilm formation) to 20 (0% of biofilm formation). It reflects the potential mobility of beads under the magnetic field applied. Three experiments with three repeats each (3 wells per slide) were performed per strain and incubation time.

To study the impact of different conditions tested on biofilm formation (glucose, antibiotics), we performed the experiments in BHI (Brain Heart Infusion) medium and in BHI medium with glucose and antibiotics.

Statistical analysis

Statistics and graphs were prepared using the software package (GraphPad Prism 6.0). For the nematode killing assays, differences in survival rates between the different strains were tested by a log-rank (Mantel-cox) test for statistical significance.

Log-transformed data were used for real time RT-PCR. The effects of medium in virulence genes and regulator of *S. aureus* were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. $P < 0.01$ was considered to reflect a statistically significant difference.

Results

Virulence of clinical *S. aureus* strains after 24h: model of acute infection

To examine the effect of high level of antibiotics (0.75 MIC), glucose (10%), glucose+vancomycin (GV) and glucose+linezolid (GL) and anaerobic conditions on *S. aureus* virulence, three *S. aureus* infecting

strains (NSA739, 1077, 7475) and one colonizing strain (NSA1385) were cultured in those medium. qRT-PCR analysis, phenotypic test and assays in *C.elegans* were performed to see the impact of those conditions in *S. aureus* virulence.

Results are summarised in Table 3.

***Vancomycin exposure:** After 24h in vancomycin, significant up-regulation of virulence factors was observed: *sea* (2.93) and *spa* (2.79) in NSA739; *lukS/lukF-PV* (2.28) and *sea* (3.37) in 1077. However, vancomycin negatively affected expression of *hla* (-2.61) in 1077, *sea* (-2.23) and *spa* (-4.06) in 7475, *fnb* (-2.28) in NSA1385. Vancomycin has no effect on the expression of the global regulator *agr* of the infecting strains NSA739, 1077, 7475 but it induces upregulation of *agr* in the colonizing strain NSA1385. For all those strains, phenotypical test showed high expression of beta hemolysin in vancomycin as observed in the Figure 2. Infection using *C. elegans* model showed no influence on the virulence of those strains after 24h in vancomycin condition compared to the control condition LB (Table 4).

***Linezolid exposure:** After an exposure of 24h, linezolid upregulated *sea* (2.58), *eap* (2.34) in 739 and *lukS/lukF-PV* (12.45) in 1077. However, linezolid significantly decreased expression of *spa* (-2.38) in 739, *spa* (-3.34), *edinB* (-2.26), *hla* (-2.65) in 1077, *hla* (-3.74), *eap* (-7.49), *fnb* (-4.52) and *spa* (-6.06) in NSA1385. No effect of linezolid was observed in the virulence genes of 7475. Linezolid induced upregulation of *agr* in toxic strains (1077 and 7475) while no modification was observed in NSA739 and NSA1385. For all those strains, phenotypical test showed high expression of beta hemolysin in linezolid as observed in the Figure 2. Infection using *C. elegans* model in linezolid showed no effect in the virulence of 1077 and NSA739 while increase of virulence of 7475 was observed in linezolid compared to the condition control LB (LT50 4.48+/-0.5 days in linezolid and 5.89+/-0.5 in LB; p<0.001) (Table 4). For the colonizing strain NSA1385, short exposure to linezolid reduced the virulence of this strain in *C. elegans* (LT50 6.12 +/- 0.5 days in linezolid vs 5.27+/- 0.5 days in LB) while no effect of vancomycin was observed (Table 4).

***Glucose exposure:** qRT-PCR analysis revealed at 24h high impact on the expression of the different genes tested. For all the strains, except 1077, the virulence genes were downregulated suggesting an important impact of the glucose. Interestingly, the behaviour of the strain 1077 was completely different with a high impact on expression of *lukS/luF-PV* (8.12) and *edinB* (10.37) and the regulator *agr* (9.41).

Concerning the colonizing strain NSA1385, high level of glucose has no impact in the genes tested. For all the studied strains, phenotypical test showed high expression of beta hemolysin as observed in the Figure 2. Infection using *C. elegans* model showed no impact in the virulence of those strains (LT50 in glucose: 4.5 to 5.9+/-0.5 days) compared to LB after 24h (LT50 in LB: 4.5 to 5.9+/-0.5 days) (Table 4).

***High level of glucose added of antibiotics (linezolid or vancomycin):** Short exposure to glucose+vancomycin (GV) highly inhibited expression of *fnb* (-3,24) and *lukS/luF-PV* (-72,96) in NSA739, *edinB* (-61,60) and *spa* (-19549,07) in 1077, *edinC* (-2,42) and *eap* (-1776,2) in 7475. However, GV induces upregulation of *hla* (14,98) and *sea* (3,42) in NSA739; *hla* (8,96), *sea* (6,03) and *eap* (1904,83) in 1077; *spa* (3326,9) in 7475 and *fnb* (3,31) in NSA1385. The global regulator *agr* was upregulated in 1077 (36,37) and in NSA1385 (2,27).

In glucose+linezolid (GL), *spa* was downregulated in all the strains: *sea* (-4,12) in 7475, *hla* in NSA739 (-2,13) and NSA1385 (-6.10). Expression of *lukS/luF-PV* (3,98) was upregulated in 1077; *fnb* in NSA739 (4,51) and NSA1385 (2,17) expression were increased. *agr* was upregulated in 1077 (42,43) (Table 3). For all these strains, phenotypical test showed high expression of beta hemolysin as observed in the Figure 2. So, at 24h, it seems that the association between glucose and vancomycin mostly negatively impact virulence genes regulation of *S. aureus* strains 1077 and 7475 and GL reduces the virulence of 7475, NSA739 and NSA1385. *Caenorhabditis elegans* experiments showed no significative difference in the virulence of these strains except the reduction of the virulence of NSA739 in GL (LT50 6+/- 0.5 days in GL vs 4.45+/- 0.5 days in LB , p  0.001) (Table 4).

***Anaerobic condition** induces upregulation of some virulence factors: *hla* (6,78), *sea* (13,67), *eap* (15,99), *fnb* (10,71) for NSA739; *hla* (5,05) and *lukS/luF-PV* (6,96) for 1077; *fnb* (2,53) and *spa* (2,52) for NSA1385 (Table 3). However, in 1077 strain, anaerobic condition induces significant decrease of *edinB* (-2,54) and *spa* (-4,41). No relevant effect was observed in the expression of virulence genes in strain 7475. The regulator *agr* was upregulated in 1077 (2,70) and NSA1385 (2,51). For all these strains, phenotypical test showed high expression of beta hemolysin as observed in the Figure 2. Anaerobic condition seems to upregulate virulence genes of the strain NSA739, 1077 and 1385 while no effect was observed in 7475. However, infection using *C. elegans* model showed no influence in the virulence of those strains after 24h in anaerobic condition compared to the control condition LB (Table 4).

Virulence of clinical S. aureus strains after prolonged time exposure: chronic infection model

To try to investigate the behaviour of *S. aureus* strains in situation of chronic infection, an *in vitro* model was performed. This *in vitro* model consisting to cultivate different *S. aureus* strains every day in different stress conditions: vancomycin, linezolid, glucose, anaerobia, glucose+vancomycin (GV) and glucose+linezolid (GL). After 16 weeks and 24 weeks, we saw the apparition of small variant colony in all strains and all conditions, which are characteristic to *S. aureus* in chronic infection (Figure 2). qRT-PCR, phenotypic assays and *C. elegans* experiments were performed in infecting and colonizing strains to study the effect of long exposure to the virulence of these strains.

***Antibiotics:** Prolonged exposure time in vancomycin induced downregulation of *spa* (-2,01), *edinB* (-15,38) in 1077; *edinC* (-19,66) in 7475. However, some virulence genes were upregulated: *hla* (22,45), *sea* (3,17) for 7475; *hla* (3,7), *sea* (5,14), *fnb* (9,68), *eap* (4,48) and *spa* (4,32) for NSA739. *agr* was also upregulated (20,95) in NSA739.

In linezolid, downregulation of the main virulence genes of *S. aureus*: *lukS/luF-PV* (-12,48), *edinB* (-22,66), *hla* (-3,03) for 1077, *edinC* (-14,38) in 7475. However, *hla* (4,57) in NSA739, *sea* in 1077 (13,38), *hla* (31,31), *sea* (23,49) and *spa* (8,99) in 7475 were upregulated (Table 3). High loss of beta hemolysin production was also observed in those strains, with 100% of loss for 1077 and 40% for NSA739 after linezolid long exposure; 90% of loss for 1077 and 40% for 7475 after vancomycin long exposure. Note that the other colonies are very few hemolytic compared to the control growth in LB. *Caenorhabditis elegans* experiments revealed a significant decrease of virulence of NSA739 in antibiotics (LT50 5.51+/-0.5 days in linezolid and 5.18+/-0.5 days in vancomycin) compared to their virulence after 24h of culture (LT50 4.54+/-0.5 days in linezolid 4.48+/-0.5 days in vancomycin). Also, diminution of the virulence of 7475 was also observed in linezolid (LT50 5.78+/-0.5 days after 16 weeks vs 4.47+/-0.5 days after 24h) (Table 4).

For NSA1385, expression of *hla*, *fnb*, *spa* and *agr* was downregulated in vancomycin compared to the results obtained after 24h. In linezolid, expression of *hla*, *sea*, *fnb*, *eap*, *spa* was increased compared to the results obtained after 24h. Phenotypic experiments showed that 75% of colonies didn't express beta hemolysin in linezolid and 100% in vancomycin (Figure 2). So, it seems that long exposure to antibiotics doesn't really affect the virulence of NSA1385. This result was confirmed by nematodes experiments that showed no significative difference in the virulence of this strain after 16 weeks in vancomycin and linezolid. Those results suggest that in situation of chronic infection, vancomycin and linezolid treatment decreased the virulence of *S. aureus* strains mainly by negatively impacting the major virulence factors *lukS/luF-PV* and *edin*. However, in NSA739, all virulence factors tested were upregulated in vancomycin but this result was not confirmed in nematode model.

***Glucose:** Prolonged exposure of *S. aureus* strains in high level of glucose negatively affected the adhesion by a downregulation of *fnb* in the strain NSA739. *sea* and *edinC* were also negatively affected in 7475 and *spa* expression was downregulated in 1077. Interestingly, opposite to results obtained in 1077 at 24h, no more overexpression of *edinB*, *lukS/luF-PV* and *agr* was observed in 1077. However, long exposure to glucose increased the expression of *hla* and *eap* in NSA739 and *hla* in 7475 (Table 3).

In the colonizing strain NSA1385, long exposure to glucose induced downregulation of *fnb*, *spa* and the regulator *agr*.

Phenotypic assays showed a loss of beta hemolysin production (Figure 2): 5% in 7475, 10% in NSA739, 50% in NSA1385 and 60% in 1077. In *C. elegans* model, long exposure to glucose reduced significantly the virulence of the colonizing strain NSA1385 (LT50 6,2+/-0.5 days after 24weeks vs 5,03+/-0.5 days after 24h).

So, qRT-PCR and phenotypic assays showed that long exposure to glucose negatively impact the expression of toxin genes of strains 7475 and 1077. The same result was observed for the colonizing strain NSA1385. For NSA739, few positive and negative changes in expression of the virulence genes have been observed and nematodes assays showed no modification in the virulence.

***Glucose + vancomycin/linezolid:** Addition of antibiotics after 16weeks of culture in high level of glucose induced the increase of expression of *fnb*, *eap*, *hla* in GV and GL and decreased the expression of *spa* in GV for NSA739 in comparison to the results obtained after 16 weeks in high glucose medium. No effect was observed after addition of vancomycin in 1077 but a decrease of expression of *lukS/luF-PV*, *eap*, *spa* and an increase of *edinB* expression in GL was observed. For the strain 7475, *sea* and *spa* were highly downregulated while *edinC* and *eap* were upregulated in GV and GL in comparison to the condition with glucose after 16 weeks in culture (Table 3). The global regulator *agr* was downregulated in GV for the strains NSA739 and 7475. In GL medium, *agr* was also downregulated for 7475 but upregulated for NSA739. So, it seems that the addition of vancomycin didn't have any effect in the virulence of 1077 and this result was confirmed in *C. elegans* experiment where the LT50 of in glucose 16 weeks were no significantly different from LT50 after addition of vancomycin 8 weeks later (LT50 4.42+/-0.5 days after 16weeks in glucose vs 4.96+/-0.5 days after 8 weeks in GV) (Table 4). Loss of beta hemolysin was almost the same in glucose 16 weeks (60%) and GV (50%). The addition of linezolid reduced the virulence of this strain by reducing expression of virulence genes, 100% of loss of beta hemolysin production in GL and this result was also confirmed in *C. elegans* experiment (LT50 4.42+/-0.5 days after 16weeks in glucose vs 5.4+/-0.5 days after 8 weeks in GL) (Table 4).

***Anaerobic condition:** in long exposure to anaerobic condition, overexpression of *hla*, *eap*, *spa* was observed in NSA739. Significant decrease of the expression of the main virulence factors (*lukS/luF-PV*, *edinB*, *edinC*) of strains 1077 and 7475 was observed. However, expression of some others virulence factors was still stimulated in these strains: *sea*, *spa* for 1077 and *hla*, *spa* for 7475 (Table 3). Assays in *C. elegans* showed no significant difference in the virulence of those strains in anaerobic after 16 weeks (Table 4). The phenotypic assays showed the loss of beta hemolysin in those strains in anaerobic condition (99%, 100% and 92% of non hemolytic colonies for NSA739, 1077 and 7475 respectively) demonstrating the high impact of anaerobic condition in virulence of those strains.

Effect of glucose and vancomycin on biofilm formation

qRTPCR results showed interesting variations of the expression of *eap*, one of the gene responsible for the biofilm formation in *S. aureus* in glucose and subinhibitory concentration of vancomycin media. At 24h, *eap* was upregulated in GV for 1077 and downregulated in GV for 7475. After 16 weeks, this gene was upregulated in glucose and vancomycin for NSA739 strain and at 24 weeks, it was upregulated in GV for NSA739 and 7475 strains (Table 3). Based on these results, we performed assays on biofilm formation at different time point. Results were represented in Table 5. After 1h30 of incubation, biofilm formation was maximal (100%) for NSA739 and 7475 strains, and partially formed for 1077 (71,6%).

***Vancomycin:** For NSA739, a punctual exposure of vancomycin had a low impact on biofilm formation (1h30 post incubation) with 80.1% of biofilm formed. However, a prolonged exposure of vancomycin had an impact on this strain only 36.6% of biofilm was constituted after 1h30. For 1077

and 7475 strains, a punctual exposure of vancomycin had no impact on biofilm formation (1h30 post incubation): 100% of biofilm was formed. However, a prolonged exposure of vancomycin had an impact on these strains because only 8.4% and 72.9% of biofilm was formed after 1h30 for 1077 and 7475 respectively. So, short exposure to vancomycin accelerated biofilm formation for 1077 and decreased it for NSA739. Prolonged exposure to vancomycin decreased biofilm formation in all the studied strains.

***Glucose:** Short exposure in glucose induced decrease of biofilm formation (1h30 post incubation) in all the strains: no biofilm for NSA739 and 1077, 8,2 % for 7475. Long exposure to high level of glucose decreased biofilm formation for NSA739 (90,9% in glucose 24h and 65,1% in glucose 16weeks, 3h post incubation) while it was increased in 7475 (8,2% in glucose 24h vs 48,4% in glucose 16 weeks (1h30 post incubation)). No difference was observed in biofilm formation between short and long exposure for the strain 1077. So, high level of glucose induced a decrease of biofilm formation. Interestingly, addition of vancomycin in medium with high level of glucose after 16 weeks has induced biofilm formation for all the strains: 0% in glucose 16 weeks vs 100% at 24 weeks in GV for NSA739 and 1077; 48,4% in glucose 16 weeks vs 53,4% at 24weeks in GV for 7475.

Discussion

DFU represents a particular environment: high level of glucose in the blood, arteriopathy, hypoxia and ischemia that induces less of oxygenation of tissues. Treatment of DFI requires the establishment of antibiotics leading to a constant impregnation of tissues by antibiotics. Moreover, the arteriopathy could decrease the distribution of antibiotics on the foot. So, in the aim to determine the real impact of this environment on the virulence of *S. aureus* in this infection, we developed an *in vitro* model. In this model, some conditions encountered in DFU were reproduced: anaerobic condition, high glucose and subinhibitory concentration of antibiotics.

Virulence of clinical S. aureus strains after punctual exposure: model of acute infection

The results obtained after 24h of exposure at different conditions miming a situation of acute infection showed upregulation of *lukS/luF-PV* in glucose, in anaerobic condition, in vancomycin and in linezolid. However, Otto *et al.*²⁰ have highlighted a reduction of Pvl production at the concentrations of 0.25 to 0.5 MIC after 4 and 6 h of incubation whereas vancomycin showed no relevant effect. These differences in the result could be explained by different times of incubation and different concentrations of linezolid used. In fact, Dumitrescu *et al.*²⁷ have shown that linezolid can have different effect depending on the strain and the concentration. For the first time, the impact of the association between glucose and antibiotics on *lukS/luF-PV* expression was studied. GV downregulated *lukS/luF-PV* while it was upregulated in GL. Interestingly, all the conditions except GV highly stimulated the expression of *lukS/luF-PV* and increased the virulence in 1077 strain.

EdinB was also upregulated in glucose but was negatively impacted in anaerobia (-2,54), linezolid (-2,26) and GV (-61,60). On the other hand, other virulence factors were also highly expressed in this strain: *hla* (5,05) in anaerobia and *sea* (3,37) in vancomycin but as previously described²³.

Those results were similar to the results obtained for the infecting strain NSA739 after 24h of exposure. In this strain, virulence factors were also upregulated in vancomycin (*sea,spa*), linezolid (*sea,eap*), anaerobic condition (*hla,sea,eap,fnb*), GV (*hla,sea*) and therefore these conditions induced an increase of virulence of NSA739. However, Bernardo *et al.*²⁸ have shown downregulation of *sea* at 0.9X MIC of linezolid. On the other hand, in this strain, *hla* expression was not modified by the linezolid while others authors have shown downregulation of *hla* at 0.9X MIC²⁸ and 0.5X MIC²⁰. In

accordance to these two previously studies ^{20,28}, we found that *spa* was downregulated after linezolid exposure.

For the strain 7475, no change of expression of tested genes have been found after punctual exposition at different stress conditions except downregulation of *edinC* in GV. Thus, on the contrary to the PVL strain 1077, all these conditions seem to not decrease the virulence of the strain 7475 except in GV condition where the decrease of virulence in nematode is correlated to the downregulation of *eap* and *edinC*. The analysis of our results doesn't allow to conclude as already been published in the past that glucose induces downregulation of virulence factors in *S. aureus* after 6h of incubation ²⁹.

Virulence of clinical S. aureus strains after prolonged time exposure: chronic infection model

After prolonged time exposure, major virulence genes as *lukS/luF-PV*, *EdinB* and *EdinC* were downregulated particularly after vancomycin and linezolid exposure. This result confirms the importance of the use of these antibiotics in DFU infection to fight against *S. aureus*. In this study, linezolid has been shown to be more efficient than vancomycin to reduce expression of PVL and EDIN. This finding is in adequation with the role of linezolid that has been shown to be highly efficient with PVL-producing *S. aureus* ²¹. Authors have been previously shown ^{21,30,31} that linezolid has a better clinical action than vancomycin specially for long treatment (over 28 days). However, results obtained with *C. elegans* model have been showed that this decrease of expression of virulence factors didn't have impact on the virulence of the *S. aureus* strains.

On the other hand, an increase of the virulence of 1077 and 7475 strains in presence of high glucose, after 16 weeks, has been highlighted in *C. elegans* experiments. Supporting this information, other works have shown that hyperglycemic conditions inhibit complement-mediated opsonization of *S. aureus*, anaphylatoxin and thus increase the severity of the *S. aureus* infections ^{22,32}. In this study, the presence of glucose increased the expression of the toxin *hla* in NSA739 and 7475 but no modification in the level expression were observed in the enterotoxin *sea* in all the strains after 16 weeks in comparison to 24h.

Effect of glucose and vancomycin in the biofilm formation

Concerning the biofilm formation, it was decreased in long exposition to vancomycin for all the strains while qRT-PCR showed upregulation of *eap* for NSA739 and no modifications for 1077 and 7475 strains. In glucose, the biofilm formation was also slowed and *eap* was upregulated in NSA739. On the contrary, the production of biofilm was accelerated in 7475 after 16 weeks of exposure of glucose while no change was observed in the *eap* expression in comparison to 24h. Those differences between the variation of *eap* gene and the biofilm formation can be explained by the fact that *eap* is not the only gene responsible for biofilm formation. Others proteins can affect the biofilm: polysaccharide intercellular antigen (PIA)³³ and FnbpA et B ³⁴. For example, O'neil *et al.* ³⁴ have shown that FnbpA and B were involved in biofilm development of meticillin-susceptible *S. aureus* (MSSA). Results obtained for the strain NSA739 have shown that *fnbA* was downregulated after 16 weeks in high level of glucose and that these results were correlated to the decrease of biofilm formation. The addition of vancomycin in medium with high concentration of glucose after 16 weeks induced biofilm formation for all the strains. It was correlated with the increase of *eap* expression in GV and GL for NSA739 and 7475.

So, it appears that *S.aureus* virulence is highly impacted after 16 weeks in all those conditions primarily by the inhibition of the expression of major virulence factor *lukS/luF-PV*, *edinB* and *edinC*. However, *S. aureus* strains try to survive in these stringent environments of DFU by activation of expression of others virulence factors like toxins *sea*, *hla*, *hly*, by mutation to small colony variant or by biofilm formation. This result was confirmed by *C. elegans* experiments that have shown after 16 and 24 weeks those strains were still virulent compared to OP50.

Concerning the strain 1077, the global regulator *agr* was upregulated in all conditions except for vancomycin, after 24h of exposure. It could explain the overexpression of several virulence factors: *lukS/luF-PV* in glucose, linezolid, anaerobic condition and GL; *hla* in anaerobic condition and GV; *edinB* in glucose; *eap* in GV. On the other hand, *spa* was downregulated in all the conditions except for vancomycin. Those results are in adequation with others studies ^{11,28,29,35,36} : *agr* upregulates *sea*, *eap*, *hla*, *lukS/luF-PV* and downregulates *fnb*, *spa*. The results obtained after 16 or 24 weeks have shown that the expression of *agr* was not modified except in anaerobic condition. Those results are in the adequation with other study ³⁷ that showed that *agr* is required in acute infection to promote virulence of the bacteria while in chronic infection, it does not interfere to allow bacterial persistence ³⁷. Concerning the strains NSA739 and 7475, the changes of *agr* expression were not correlated with the variation of expression of virulence genes potentially regulated by *agr*. This result can be explain by the fact that *agr* is not the alone regulator in *S. aureus*. All those genes are also regulated by others regulators such as *rot*, *saeR*, *sar*, *sigB* ^{14,18,36,38}. Moreover, *agr* also can be positively regulated by members of *sar* family ^{18,38,39} and negatively regulated by *sigB* ¹⁸. The apparition of SCV in those conditions after 16 and 24 weeks validates this model as a good model to study persistant infection of *S. aureus*.

Conclusion

In this study, for the first time the impact of conditions encountered in DFU were studied on a long time. We observed that antibiotics (linezolid and vancomycin), glucose and anaerobic condition can negatively affect the virulence of those strains. However, in these conditions, *S. aureus* try to survive by activation of others virulence mechanisms. This study allowed a better understanding of the behaviour of *S. aureus* in DFU.

Acknowledgments

Fer-15 nematodes were provided by the Caenorhabditis Genetics Center, a foundation of the NIH National Center for Research Resources (NCRR).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

The work was supported by INSERM.

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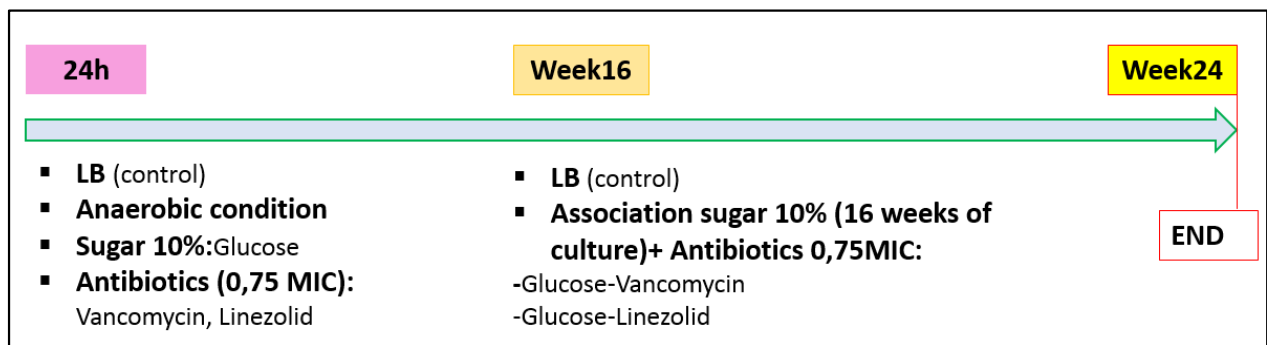
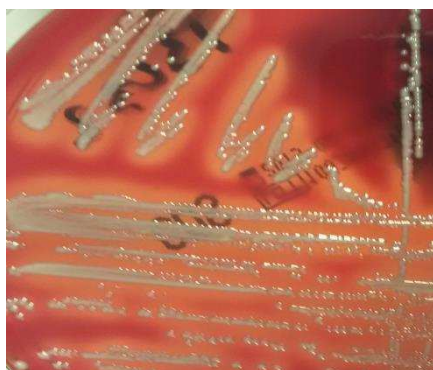
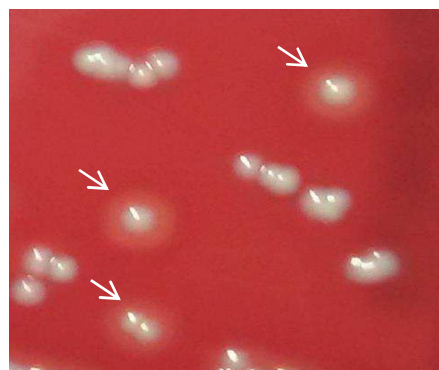


Figure 1: Different conditions used in this study.

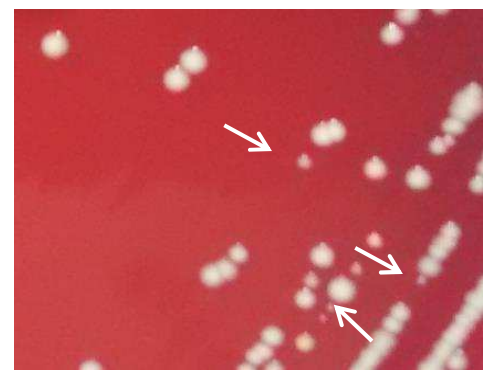
Cultures were made each day in anaerobic condition, glucose 10% and antibiotics at 0.75 MIC (linezolid and vancomycin. After 16weeks, antibiotics cultures were stopped and antibiotics were added in glucose 16 weeks culture. 8weeks later, experiment was stopped.



A. Strain producing beta hemolysin



B. Strains with few (indicate by arrows) or non expression of beta hemolysin



C. Arrow indicate Small Colony variants

Figure 2: View of *S. aureus* culture in blood agar gelose.

- S. aureus* colonies expressing beta hemolysin. Gelose becomes very clear indicating the lysis of hemolysins.
- Some *S. aureus* colonies expressed few beta hemolysin (visualise here by a low lightening of agar around the colonies compared to A) and the others doesn't express beta hemolysin.
- Visualisation of Small Colony variants after 16 weeks of culture.

Table 1. *S. aureus* strains used in this study

Strains/Plasmids	Characteristics	References
Newman	Reference strain	-
NSA739	<i>S. aureus</i> strain isolated from infected diabetic foot ulcer (Grade 3) PVL-, CC5	(Sotto <i>et al.</i> , 2008)
NSA1385	<i>S. aureus</i> strain isolated from colonized diabetic foot ulcer (Grade 1), PVL-, agr1, CC8	(Sotto <i>et al.</i> , 2008)
1077	<i>S. aureus</i> strain isolated from infected diabetic foot ulcer (Grade 3), PVL+, edinB+	Messad <i>et al.</i> , 2012
7475*	<i>S. aureus</i> strain isolated from infected diabetic foot ulcer (Grade 3), PVL-; edinC+	Messad <i>et al.</i> , 2012)

Table 2 Primers used in the study

TARGET FUNCTION	TARGET REGION	PRIMER NAME	OLIGONUCLEOTIDE SEQUENCE	TM (°C)	REFERENCE
TOXINS	<i>hla</i>	hla- F	5'- TCCAGTGCAATTGGTAGTCA -3'	55.3	20
		hla- R	5'- GGCTCTATGAAAGCAGCAGA-3'	57.3	
	<i>sea</i>	sea- F	5'- ATGGTGCTTATTATGGTTATC -3'	52.0	40
		sea- R	5'- CGTTTCCAAAGGTACTGTATT -3'	54.0	
	<i>pvl</i>	lukS F	5'- AATAACGTATGGCAGAAATATGGAATGT-3'	58.9	36
		lukS R	5'- CAAATGCGTTGTGTATTCTAGATCCT-3'	60.1	
	<i>edinB</i>	edinB-F			
		edinB-R			
	<i>edinC</i>	edinC-F			
		edinC-R			
ADHESION	<i>fnbA</i>	fnbA- F	5'- AAATTGGGAGCAGCATCAGT -3'	55.3	41
		fnbA- R	5'- GCAGCTGAATTCCCAATTTTC -3'	55.3	
	<i>eap</i>	eap- F	5'- AAGCGTCTGCCCGCAGCTA -3'	58.2	12



ESCAPE		eap- R	5'- TGCATATGGAACATGGACTTTAGAA -3'	58.1
	<i>spa</i>	spa-F	5'- TATGCCCTAACTTAAATGCTG -3'	51.1 42
		spa- R	5'- TTGGAGCTTGAGAGTCATTA -3'	53.2
REGULATOR		agrA-F	5'- CAAAGAGAAAAACATGGTTACCATTTATTAA -3'	58.2 43
	<i>agrA</i>			
		agrA-R	5'- CTCAAAGCACCTCATAAGGATTATCAG -3'	61.6
HOUSEKEEPING GENES		gyrB-F		
	<i>gyrB</i>	gyrB-R		
		HU-F	5'- CAAAGAGAAAAACATGGTTACCATTTATTAA -3'	58.2 43
		HU-R	5'- CTCAAAGCACCTCATAAGGATTATCAG -3'	61.6
	<i>HU</i>			



Table 3: qRT-PCR results: fold change of RNA levels of target genes in different cultures conditions after 24h,16 weeks and 24 weeks in comparison to LB culture at 24h, 16 weeks and 24 weeks.

	Glucose		Anaerobia		Vancomycin		Linezolid		Gluco-vanco		Gluco-linez	
	24h	16weeks	24h	16weeks	24h	16weeks	24h	16weeks	24h	24weeks	24h	24weeks
Newman												
hla	-3,24	1,66	2,14	-1,46	1,58	-4,74	-3,75	-2,13	1,11	27,64	-2,99	7,23
sea	-3,03	-1,85	4,26	1,43	1,36	-1,29	1,35	1,29	2,74	-1,77	1,47	-1,03
eap	-1,86	1,42	6,62	1,17	1,63	-1,73	-1,14	1,28	4,11	-1,12	5,03	3,03
fnb	-1,85	1,15	15,10	-1,43	2,17	-2,21	18,13	1,63	2,73	6,09	7,50	4,23
spa	-84,46	-3,86	-1,23	2,59	2,85	6,96	-31,38	3,55	-9,71	-14,44	-48,08	-4,74
agr	-2,30	-13,69	1,56	-4,87	2,07	2,00	-5,55	-1,78	1,28	-28,22	-1,03	-23,69
NSA739												
hla	-2,22	3,07	6,78	4,46	-1,60	3,72	1,37	4,57	14,98	11,67	-2,13	5,24
sea	-1,27	-1,89	13,67	1,85	2,93	5,14	2,58	1,37	3,42	1,21	-1,08	-1,09
eap	-1,86	2,04	15,99	15,64	1,19	9,68	2,34	1,78	-1,47	3,34	-1,61	3,60
fnb	-1,44	-4,15	10,71	-2,10	1,86	4,48	1,95	-1,05	-3,24	3,58	4,51	5,52
spa	-2,20	-1,39	-1,17	2,23	2,79	4,32	-2,38	1,25	1,84	-4,61	-7,85	1,05
agr	-7,84	-1,82	-1,06	-2,27	-1,25	20,65	-1,98	-2,31	-1,00	-4,49	-1,27	14,78

spa	-1,70	-2,77	2,52	20,15	-1,07	-4,06	-6,06	-2,98	-1,31	-4,19	-3,62
agr	1,67	-147,42	2,51	-13,67	3,79	1,08	-1,36	1,17	2,27	-1,06	-33,90

Table 4: 50% Lethal Time of *Caenorhabditis elegans* infected by *S. aureus* strains NSA 1385, NSA739, 1077, 7475, Newman and evaluation of feeding behaviour by measuring the pathogen avoidance. The results are representative of at least four independent trials for each group of strains. P: Pairwise comparison between LT50s using a log rank test. NS: not significant. LT50: 50% Lethal Time. Differents conditions: G: Glucose 10%, V: Vancomycin 0,75 MIC, L: Linezolid 0,75 MIC, A: Anaerobia, GV: Glucose 10%+ Vancomycin 0,75 MIC, GL: Glucose 10%+ Linezolid 0,75 MIC.

LT50 (in days)		p	p	p	p
		OP50 vs others	24h vs 16 or 24 weeks	24h vs LB 24h	16weeks G vs GV or GL
OP50	7,557	-			
24h					
1385 LB ON	5,27	<0,001	-	-	
1385 G ON	5,03	<0,001	<0,001	NS	
1385 V ON	5,275	<0,001	NS	NS	
1385 L ON	6,116	<0,001	NS	<0,001	
1385 A ON	5,427	<0,001	NS	NS	

1385GV ON	5,087	<0,001	NS	NS
1385 GL ON	5,40021	<0,001	NS	NS
16 weeks				
1385 LB 16W	6,0649	<0,001	NS	-
1385 G 16W	6,201	<0,001	<0,001	NS
1385 V 16 W	6,083	<0,001	NS	NS
1385 L 16W	6,563	<0,001	NS	NS
1385 A16W	6,165	<0,001	NS	NS
24 weeks				
1385LB 24W	5,807	<0,001	NS	-
1385 GV 24W	5,661	<0,001	NS	NS
1385 GL 24W	5,938	<0,001	NS	NS
24h				
Newman LB ON	4,888	<0,001	-	-
Newman G ON	5,244	<0,001	NS	NS
Newman V ON	5,437	<0,001	NS	NS
Newman L ON	5,273	<0,001	NS	NS
Newman A ON	4,943	<0,001	NS	NS
Newman GV ON	4,87	<0,001	<0,001	NS
Newman GL ON	5,606	<0,001	NS	NS



16 weeks					
Newman LB 16V	5,555	<0,001	NS	-	
Newman G 16V	5,603	<0,001	NS	NS	
Newman V 16V	5,547	<0,001	NS	NS	
Newman L 16V	5,815	<0,001	NS	NS	
Newman A 16V	5,431	<0,001	NS	NS	
24 weeks					
Newman LB 24W	6,045	<0,001	<0,001	NS	
Newman GV 24W	5,881	<0,001	<0,001	NS	NS
Newman GL 24W	5,999	<0,001	NS	NS	NS
24h					
739 LB ON	4,454	<0,001	NS	-	
739 G ON	4,497	<0,001	NS	NS	
739 V ON	4,482	<0,001	<0,001	NS	
739 L ON	4,544	<0,001	<0,001	NS	
739 A ON	4,438	<0,001	NS	NS	
739 GV ON	4,935	<0,001	<0,001	NS	
739 GL ON	6	<0,001	NS	<0,001	
16 weeks					
739 LB 16W	4,988	<0,001	NS	-	



739 G 16W	4,934	<0,001	NS	NS
739 V 16W	5,183	<0,001	<0,001	NS
739 L 16W	5,508	<0,001	<0,001	NS
739 A 16W	5,049	<0,001	NS	NS
24weeks				
739 LB 24W	5,938	<0,001	<0,001	-
739 GV 24W	5,898	<0,001	<0,001	NS
739 GL 24W	5,968	<0,001	NS	<0,001
24h				
7475 LB ON	5,887	<0,001	-	-
7475 G ON	5,898	<0,001	<0,001	NS
7475 V ON	5,754	<0,001	NS	NS
7475 L ON	4,477	<0,001	<0,001	<0,001
7475 A ON	5,698	<0,001	NS	NS
7475 GV ON	5,814	<0,001	NS	NS
7475 GL ON	6,206	<0,001	NS	NS
16 weeks				
7475 LB 16 W	5,391	<0,001	NS	-
7475 G 16 W	4,477	<0,001	<0,001	<0,001
7475 V 16 W	5,732	<0,001	NS	NS



7475 L 16 W	5,783	<0,001	<0,001	NS
7475 A 16 W	5,649	<0,001	NS	NS
24 weeks				
7475 LB 24W	5,983	<0,001	NS	NS
7475 GV 24W	5,509	<0,001	NS	<0,001
7475 GL 24W	5,972	<0,001	NS	NS
24h				
1077 LB ON	5,903	<0,001	-	-
1077 G ON	5,169	<0,001	<0,001	NS
1077 V ON	5,546	<0,001	NS	NS
1077 L ON	5,546	<0,001	NS	NS
1077 A ON	6,28	<0,001	NS	NS
1077 GV ON	5,888	<0,001	<0,001	NS
1077 GL ON	5,412	<0,001	NS	NS
16 weeks				
1077 LB 16W	5,428	<0,001	NS	-
1077 G 16W	4,422	<0,001	<0,001	<0,001
1077 V 16W	5,673	<0,001	NS	NS
1077 L 16W	5,456	<0,001	NS	NS
1077 A 16W	5,847	<0,001	NS	NS



24 weeks				
1077 LB 24W	5,439	<0,001	NS	-
1077 GV 24W	4,96	<0,001	<0,001	NS
1077 GL 24W	5,424	<0,001	NS	<0,001

Table 5: Biofilm formation

Percentage of biofilm formation of strains NSA739, 1077, 7475 in Glucose 16 weeks, Vancomycin, Glucose+Vancomycin after 24h, 16 weeks, 24 weeks. Value represent mean of three different experiments.

Strains	BHI	Glucose 24h		Glucose 16 weeks		Vancomycin 24h		Vancomycin 16 weeks		Gluco-Vanco 24h		Gluco-Vanco 24weeks	
		1h30	3h	1h30	3h	1h30	3h	1h30	3h	1h30	3h	1h30	3h
NSA739	100	0	90,9	0	65,16	80,14	100	36,58	94,51	100	100	100	100
1077	71,62	0	100	0	100	100	100	8,44	100	94,83	100	100	100
7475	100	8,24	100	48,41	100	100	100	72,92	100	72,09	100	53,42	100



Travail n°5

Attenuation of *Staphylococcus aureus* virulence by *Helcococcus kunzii* in a *Caenorhabditis elegans* model

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Article original : Soumis à publication « Frontiers cellular and infection microbiology »

OBJECTIF : Etudier sur le modèle *in vivo* *C. elegans* l'effet de l'association du *S. aureus* avec une autre bactérie commensale non pathogène fréquemment isolée du pied diabétique, *Helcococcus kunzii*, sur la virulence des souches de *S. aureus*.

RESUME :

L'infection du pied diabétique est majoritairement polymicrobienne et *S. aureus* est la principale bactérie mise en cause. Récemment, il a été montré que *S. aureus* était fréquemment isolé en association avec *H. kunzii*, un cocci à Gram positif, catalase-négatif, anaérobie facultatif^{106–109}. Le rôle de cette bactérie étant inconnu dans les infections polymicrobiennes, nous avons décidé d'étudier l'impact de la co-infection sur la virulence du *S. aureus* et d'*H. kunzii* en utilisant le modèle *Caenorhabditis elegans*.

Pour ce faire, nous avons travaillé sur 23 souches cliniques d'*H. kunzii* isolées de plaies de pieds chez des patients diabétiques, deux souches cliniques de *S. aureus* ainsi que la souche de référence Newman. La virulence de ces souches seules ou en association a été évaluée sur le modèle *C. elegans*. Nous avons déterminé le temps de demi-vie des nematodes (LT50 : correspondant au temps nécessaire pour que la moitié des nematodes soit mort), évalué le nombre de bactéries ingérées par le nématode et se trouvant dans leur tube digestif et étudié l'effet toxique des souches en évaluant le comportement des nematodes. Des études transcriptomiques par qRT-PCR ont été faites sur le nématode en quantifiant l'expression de 6 gènes de virulence importants : *hlh-30* (facteur de transcription clé codant pour les gènes de défense de l'hôte), *lys-5* et *clec-7* (gènes antimicrobiens), *cyp-37B1* et *sodh-1* (gènes de cytoprotection et de détoxification) et *lgg-1* (gène de l'autophagie). L'expression de deux gènes de virulence du *S. aureus* (la protéine A, *spa* et l'alphatoxine, *hla*) ainsi que du régulateur global, *agr* ont également été évalué par qRT-PCR.

Les résultats ont montré que les souches d'*H. kunzii* testées n'étaient pas virulentes (LT50: 6.9 jours $\pm$ 0.5) ou étaient très peu virulentes (LT50: 5.7 jours $\pm$ 0.4). Par contre, les souches de *S. aureus* (LT50: 2.9 jours $\pm$ 0.4) étaient significativement plus virulentes que les souches d'*H. kunzii* ($p < 0.001$). Les études transcriptomiques ont confirmé ces résultats en montrant que l'ingestion des souches de *S. aureus* contrairement à celles d'*H. kunzii* ont conduit à l'activation des gènes de défense de l'hôte (*lys-5*, *sodh-1* et *cyp-37*).

Lorsque les souches de *S. aureus* et d'*H. kunzii* étaient associées, les souches d'*H. kunzii* réduisaient significativement la virulence des souches de *S. aureus* dans le modèle du

nématode en augmentant leur durée de vie (LT50 entre 4.4 et 5.2 jours ; $p < 0.001$). Aucune différence significative n'a été observée dans l'expression des facteurs de virulence de l'hôte quand ils étaient nourris avec les souches de *S. aureus* seules ou en association avec *H. kunzii*. Cependant, les études d'infection séquentielles et les études transcriptomiques sur les souches de *S. aureus* nous ont montré que *H. kunzii* affectait la virulence de *S. aureus* par interaction directe en affectant l'expression des gènes de virulence notamment une légère diminution d'expression du régulateur global et une légère surexpression de la protéine A.

Cette étude a donc montré une réduction de la virulence des souches de *S. aureus* par les souches d'*H. kunzii*. Des études ultérieures doivent encore être effectuées pour comprendre les mécanismes de réduction de cette virulence.

Attenuation of *Staphylococcus aureus* virulence by *Helcococcus kunzii* in a *Caenorhabditis elegans* model

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Running title *H. kunzii* and *S. aureus* cooperation



Abstract

Social bacterial interactions are considered essential in numerous infectious diseases, particularly in wounds. Foot ulcers are a common complication in diabetic patients and these ulcers become frequently infected. This infection is usually polymicrobial promoting cell-to-cell communications. *Staphylococcus aureus* is the most prevalent pathogen isolated. Its association with *Helcococcus kunzii*, commensal Gram-positive cocci, is frequently described. The aim of this study was to assess the impact of co-infection on virulence of both *H. kunzii* and *S. aureus* strains in a *Caenorhabditis elegans* model. To study the host response, qRT-PCRs targeting host defense genes were performed. We observed that *H. kunzii* strains harboured a very low (LT50: 5.7 days $\pm$ 0.4) or an absence of virulence (LT50: 6.9 days $\pm$ 0.5). In contrast, *S. aureus* strains (LT50: 2.9 days $\pm$ 0.4) were significantly more virulent than all *H. kunzii* ($P < 0.001$). When *H. kunzii* and *S. aureus* strains were associated, *H. kunzii* significantly reduced the virulence of the *S. aureus* strain in nematodes (LT50 between 4.4 and 5.2 days; $P < 0.001$). To evaluate the impact of these strains on host response, transcriptomic analysis showed that the ingestion of *S. aureus* led to a strong induction of defense genes (*lys-5*, *sodh-1* and *cyp-37B1*) while *H. kunzii* did not. No statistical difference was observed when *C. elegans* were infected with either *S. aureus* alone or with *S. aureus* + *H. kunzii*. This study showed that *H. kunzii* decreased the virulence of *S. aureus* without modifying directly the host defense response. Factor(s) modulating the staphylococci virulence must be investigated.

Keywords: Attenuation, *Caenorhabditis elegans*, co-infection, *Helcococcus kunzii*, *Staphylococcus aureus*, virulence

Introduction

Diabetes mellitus is a worldwide public health problem representing the third cause of mortality and morbidity in the world (WHO, 2014). Foot ulcers are a common complication in diabetic patients during their life (15-25%) (Boulton *et al.*, 2005). Infection of these ulcers is a frequent complication (>50%). It represents a major cause of hospitalization, morbidity and mortality. It is also one of the major cause of lower-limb amputation (Mayfield *et al.*, 1998). Several studies have shown that diabetic foot ulcers (DFU) are polymicrobial. However *Staphylococcus aureus* represents the most frequent pathogen isolated (Commons *et al.*, 2015; Gardner *et al.*, 2013; Hatippoglu *et al.*, 2016; Lesens *et al.*, 2015; Messad *et al.*, 2013). This Gram-positive coccus is a leading cause of a wide range of diseases from skin and soft tissue infections (e.g. impetigo, carbuncles) to life-threatening bacteraemia, toxic shock syndrome, endocarditis, and osteomyelitis (Lowy, 1998), for which it deploys an arsenal of virulence factors to destroy host immune cells and tissues (Tacconelli *et al.*, 2006).

In DFU, *S. aureus* is associated with a great diverse community of bacterial species (Gardner *et al.*, 2001). Social bacterial interactions are considered essential in numerous infectious diseases, including chronic wounds. These interactions have been described in all living entities (Brodgen *et al.*, 2005). For example, a model of synergistic effect between uropathogenic *Escherichia coli* and *Enterococcus faecalis* showed that *E. faecalis* increased the virulence of *E. coli* (Lavigne *et al.*, 2008). Moreover, translocation of several enterobacteria isolates in the bloodstream results in higher mortality (Pittet *et al.*, 1993). Interactions involving clonal or divergent strains of the same species have also been described (Parsek and Greenberg, 2005; Tourret *et al.*, 2011). However, this type of documentation of bacterial interaction is scarce in DFU. If metagenomic technologies have determined that distinct communities of bacteria are present at different sites of the body, challenges remain in understanding the complex interplay of these different species in contributing to modify the bacterial virulence (Fernandez *et al.*, 2015; Gardner *et al.*, 2013; Price *et al.*, 2009).

Recently the emergence of new tools (e.g. mass spectrometry, pyrosequencing) in bacterial identification has highlighted the frequent association between *S. aureus* and *Helcococcus kunzii*, a catalase-negative, facultative anaerobic Gram-positive coccus in DFU (Chagla *et al.*, 1998; Haas *et al.*, 1997; Lemaître *et al.*, 2008; Park *et al.*, 2014; Riegel and Lepargneur, 2003; Vergne *et al.*, 2015). *H. kunzii* was first described as a non-pathogenic bacteria, likely member of the skin microbiome (Hass *et al.*, 1997). This species is also known as an opportunistic pathogen that causes different types of infections (endocarditis, bacteraemia, meningitis, breast abscess, wound infections, prosthetic joint, osteomyelitis) in immunosuppressed patients (diabetic patient, drug fiend, alcoholic) (Chagla *et al.*, 1998; Lemaître *et al.*, 2008; Park *et al.*, 2014; Vergne *et al.*, 2015). Nonetheless, the role of *H. kunzii* in the pathogenesis of cutaneous polymicrobial infections remains unknown. In this study, we sought to investigate the potential of virulence of *H. kunzii* strains isolated from DFU in a model of *S. aureus* induced infection of *Caenorhabditis elegans* (Irazoqui *et al.*, 2010; Szabados *et al.*, 2013; Visvikis *et al.*, 2014).

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains studied are listed in Table 1.

A collection of 23 clinical isolates of *H. kunzii* collected from DFU in a multicentre study performed between February 2008 and August 2013 was used (Vergne *et al.*, 2015). Moreover, to assess the co-infection between *H. kunzii* and *S. aureus*, in addition to the reference *S. aureus* strain Newman, two

clinical *S. aureus* strains, both isolated and characterized in our hospital, were used: NSA1385 (a colonizing strain collected from uninfected ulcer) and NSA739 (an infecting strain collected from deep DFI) (Messad et al., 2015; Sotto et al., 2008). *E. coli* OP50 was used as control for nematodes. This strain is the standard feeding strain for Fer-15 nematodes, which harbors no known virulence factors.

The different bacteria were grown in Mueller-Hinton (MH) and Luria Bertani (LB) broth or agar at 37°C except *H. kunzii* strains which were grown on Columbia agar supplemented with 5% fresh sheep blood (bioMérieux, France) under a 5% CO₂ atmosphere at 37°C during 48 h.

Pulse field gel electrophoresis (PFGE)

PFGE analysis of genomic DNA fragments of the 23 clinical isolates of *H. kunzii* was carried out after digestion with the restriction endonuclease *Sma*I, as previously published for enterococci (Bourdon et al., 2011). The electrophoresis was performed using a CHEF-DRIII apparatus (BioRad, France) and PFGE patterns were interpreted according to well-established criteria (Tenover et al., 1995).

Nematode Killing Assay

The nematode infection assay was carried out as previously described using the Fer-15 mutant line (a temperature sensitive fertility defect) (Lavigne et al., 2008). Overnight cultures of the studied bacterial strains in the nematode growth medium (NGM) were harvested, centrifuged and suspended in phosphate buffered saline solution (PBS) at a concentration of 10⁵ CFU/mL. 10 µL of these bacterial suspensions were inoculated on NGM agar plates. A ratio 1:1 was prepared during co-infection assays. To validate this ratio, we evaluated the CFU of both bacteria prior to the seeding onto agar to confirm that *S. aureus* and *H. kunzii* were present in an equal amount. The plates were incubated at 37°C for 8–10 h. Around 30 L4 stage nematodes per plate were thus seeded and then incubated at 25°C. Each day an independent reader (blind to the culture) scored each day the number of live nematode under a stereomicroscope (Leica, France).

Effect of sequential infection of *C. elegans*

C. elegans were infected with two representative *H. kunzii* strains (H10 with no virulence and H13 with a low virulence). After 12 h, 30 nematodes were transferred to NGM medium containing the different *S. aureus* strains. In the same way, to evaluate the bacterial persistence, nematodes were coinfecting with *H. kunzii* and *S. aureus*. After 12 h, 30 nematodes were transferred to NGM medium containing OP50 strain. The conditions of nematodes preparation were strictly similar to previous assays described before. Final analysis established the Lethal time 50% (LT50), which corresponds to time (in days) required to kill 50% of the worms.

Three replicates repeated five times were performed for each studied strain.

Feeding behaviour experiments

Firstly, all the studied bacterial strains were grown in LB broth media (with or without anaerobes conditions) at 25°C during 16 h. The cultures were then spotted on NGM plates. Around 30 L4 stage nematodes were deposited in the centre of the bacteria lawn. To establish the occupancy assays, the number of nematodes inside or outside each lawn was counted after overnight incubation as previously

published (Lavigne *et al.*, 2013). The results were presented in percent occupancy (number of worms in the bacterial lawn on the total number of *C. elegans*). The experiments were performed in triplicate.

Secondly, we determined the number of bacteria within the nematode gut (Garsin *et al.*, 2001 (Lavigne *et al.*, 2008). Finally, after serial dilutions, 100 μ l of the mixture were plated on LB agar medium and on Columbia agar supplemented with 5% fresh sheep blood (when the co-infection experiments were performed). The colonies (● haemolytic for *H. kunzii* and ○ haemolytic for *S. aureus*) were counted after 24 h. Three replicate assays were performed for each strain.

Effect of *H. kunzii* on *S. aureus* virulence genes expression

Analysis of the mRNA levels of *spa*, *hla* and *agr* was performed following the method previously published (Doumith *et al.*, 2009). *S. aureus* isolates and *H. kunzii* H13 were grown alone or in association in MH broth to an OD₆₀₀ of ~ 0.7. The total RNA extraction was performed using the RNeasy Mini kit (Qiagen, France) during growth and stationary stages. Purity and concentration were determined by the NanoDrop 2000 spectrophotometer (Fisher Scientific, USA). The iScript™ Select cDNA Synthesis Kit (Biorad, Hurcules, CA) was used to the synthesis of cDNA from 1 μ g of total RNA for each sample. Real-time PCR were done in a LightCycler®480 (Roche, France) using the LightCycler FastStart DNA Master^{PLUS} SYBRGreen I kit, 100 ng of cDNA and 10 pmol of target primers (Table 4). Amplifications were analyzed in triplicate from three different RNA preparations. Cycle threshold (Ct) values of the different target genes were compared with the Ct values of the house-keeping gene (*gyrB*). The Newman strain was used as control. The normalized relative expressions of the studied genes were obtained for each strain following the equation: $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (Ct_{gene} - Ct_{gyrB})_{studied\ strain} - (Ct_{gene} - Ct_{gyrB})_{control\ strain}$) (Livak *et al.*, 2001). Results obtained for each gene were log-transformed to obtain a fold change difference between strains.

Evaluation of host response by quantitative real time-PCR

For selected genes involved in *C. elegans* response against infection (*hlh-30*, *lys-5*, *lgg-1*, *clec-7* (Visvikis *et al.*, 2014), *cyp-37B1* (Irazoqui *et al.*, 2010), *sodh-1* (JebaMercy and Balamurugan, 2012)), transcript level analysis was performed by qRT-PCR following the same protocol described before. *C. elegans* were infected between 12 h with studied bacterial strains. The nematodes were then washed twice in water. Total RNA from animals was extracted by using TRIzol® RNA Isolation Reagents (ThermoFisher, France). The target primers used were presented in Table 4. The $2^{-\Delta\Delta Ct}$ method was used to analyze transcriptional changes in target genes using *snb-1* as the housekeeping control gene (Engelmann *et al.*, 2011). Data analysis was performed with the Pfaffl method (Pfaffl, 2001). Error bars indicate the standard deviation (SD) of three independent experiments.

Statistical analysis

Statistics and graphs were performed using GraphPad Prism 6.0 software.

For the nematode killing experiments, a log-rank (Mantel-cox) test was used to evaluate differences in survival rates between the different strains.

Log-transformed data were used for RT-PCR. The effects of bacterial infections on expression of selected genes involved in *S. aureus* virulence and in host response were performed using one-way ANOVA

followed by Dunnett's multiple comparisons test. A statistically significant difference was retained for $P < 0.01$.

Results

Results

Virulence of *H. kunzii* and *S. aureus* strains

The virulence of a clinical panel of 23 *H. kunzii* isolates was evaluated in a nematode model. The genetic comparison of the 23 strains showed that the isolates were not clonally related (data not shown) eliminating a clonal impact of the virulence behaviour. Out of 23, 17 (74%) *H. kunzii* strains were non virulent with a behaviour similar to the non-pathogenic *E. coli* OP50 (LT50s: 6.1 days ± 0.4 vs 7.1 days ± 0.5 , respectively), a laboratory reference strain used to feed nematodes (P value, non significant (NS)). The other six *H. kunzii* strains (H7, H10, H17a, H20, H21, H22b) were significantly more virulent than OP50 ($P < 0.001$) (Table 1).

To compare the virulence between *H. kunzii* and *S. aureus*, we used well-characterized *S. aureus* strains: the *S. aureus* strain NSA739 (collected from DFU and harbouring a high virulence potential), the *S. aureus* strain NSA1385 (collected from DFU and harbouring a low virulence potential) and the *S. aureus* reference strain Newman.^{27,28} We observed that the panel of *H. kunzii* presented significantly lower virulence than NSA739 (LT50: 2.8 days ± 0.4), NSA1385 (LT50: 4.7 ± 0.2) and Newman (LT50: 4.3 ± 0.3) ($P < 0.001$), respectively. The difference of virulence between the infecting and the colonizing strains of *S. aureus* was previously demonstrated (Table 1) (Messad *et al.*, 2015). These results confirmed that *H. kunzii* strains are low- or non-virulent bacteria.

Attenuation of *S. aureus* virulence by *H. kunzii*

When the different *H. kunzii* strains and NSA739 were used to co-infect *C. elegans*, an important attenuation of the *S. aureus* virulence was observed independently of *H. kunzii* virulence or non-virulence potential (Table 2, Figure 1). The LT50 obtained with the strains coinfection varied between 4.1 and 5.7 days. They were significantly longer than the LT50 detected with *S. aureus* strain alone (LT50: 2.8 days) ($P < 0.001$).

To confirm the role of *H. kunzii* in the attenuation of *S. aureus* virulence, we tested its effect in association with NSA1385 (colonizing strain). If the majority of *H. kunzii* strains did not present a significant modification of *S. aureus* virulence, 8 strains (H4, H6, H8, H13, H16, H17b, H22b, H23) reduced significantly the virulence of NSA1385 (LT50: 5.6 to 6.3 days; $P < 0.001$) (Table 3). Evaluating the association between the reference strain Newman and the 23 *H. kunzii*, we observed that 6 *H. kunzii* strains (H9, H13, H18, H22a, H22b, H23) reduced the virulence of the *S. aureus* strain (LT50: 5.8 to 7.1 days; $P < 0.001$) (Table 3).

All these findings strongly suggest the important role of *H. kunzii* strains in the attenuation of *S. aureus* virulence isolated from wounds in particular for highly virulent *S. aureus* strains.

Effect on feeding behaviour

To exclude the possibility that the observed results in worms was due to a modification of their feeding behaviour, an occupancy test was performed. None of the bacterial strains tested alone or in association presented strong avoidance behaviour. No significant difference was noted in the fraction of nematodes on the bacterial lawn between the different associations studied (Tables 1, 2 and 3). We also measured the bacterial load in the intestine of nematodes at an early time point of infection (72 h post infection). We found that all bacteria tested alone or in association can colonize and survive in the *C. elegans* intestine (Figure 2). The number of the *H. kunzii* and *S. aureus* CFU was around 4×10^5 bacteria per nematode ($IC_{95\%} = 2.8-7.9 \times 10^5$) within the nematode intestine for each combination without statistical difference (*P* value, NS).

These results confirm the low virulence of *H. kunzii* strains and suggest that the impact on the modulation of *S. aureus* virulence observed in *C. elegans* was not due to a modification of nematodes' feeding behaviour, nor to a reduction of *S. aureus* infection rate or a hypothetical cytotoxicity effect of *H. kunzii* on *C. elegans*.

Transcriptional host response during co-infection between *H. kunzii* and *S. aureus*

To estimate the host response during co-infection between *H. kunzii* and *S. aureus*, we carried out qRT-PCRs on six representative host defense genes of nematodes after infection by *H. kunzii* and *S. aureus* alone or in co-infection: *hlh-30* (the key transcriptional factor-encoded gene for *S. aureus* host defense), *lys-5* and *clec-7* (antimicrobial-encoded genes), *cyp-37B1* and *sodh-1* (cytoprotective and detoxification-encoded genes) and *lgg-1* (autophagy-encoded gene). Of the different co-infection combinations we choose to study one *H. kunzii* isolate non virulent in nematode model and reducing the virulence of all *S. aureus* strains (H13) and one with low virulence in nematode model and that had effect exclusively on NSA739 virulence (H10).

We found that nematodes fed with the two *H. kunzii* strains did not show significant differences of expression of host defense genes compared to nematodes fed with the non-pathogenic strain OP50. This result confirms that *H. kunzii* strains do not stimulate the *C. elegans* immune response (Figure 3). On the other hand, when nematodes were fed with the three *S. aureus* strains, they significantly overexpressed *hlh-30*, *lys-5*, *sodh-1* and *cyp-37B1* compared to nematodes fed with OP50 ($P < 0.01$). Only the autophagy gene *lgg-1* had no modification of expression whatever the strain and the condition tested (*P* value, NS). Interestingly, no significant difference in the expression of host response genes could be observed between each *S. aureus* strains tested (colonizing or infecting) (Figure 3). These results confirm a nematode host response when *C. elegans* were infected with *S. aureus*.

Finally, when we co-fed nematodes with *H. kunzii* and *S. aureus* strains, we observed that the majority of host defense genes (*hlh-30*, *lys-5*, *cyp-37B1* and *sodh-1*) were overexpressed compared to OP50 or *H. kunzii* alone ($P < 0.01$). Gene expression levels were equivalent to those observed with nematodes fed with *S. aureus* alone (*P* value, NS) (Figure 4). Surprisingly the *clec-7* gene has only significant variation of expression when *C. elegans* were fed with the colonizing *S. aureus* strain NSA1385 ($P < 0.05$).

These results suggest that the co-infection with *H. kunzii* and *S. aureus* induced an overexpression of some host defense genes. However, the modulation of expression of *clec-7* gene during the coinfection of *H. kunzii* and the virulent/non virulent *S. aureus* strains could suggest some modulations of host defense. So, during the coinfection, the attenuation of *S. aureus* virulence in presence of *H. kunzii* seems to not be due

to its capacity to trigger *C. elegans* host response that would help the fight against *S. aureus* infection. *H. kunzii* seems to directly act in the modulation of *S. aureus* virulence and to have no major role on the modulation of host immune defense.

Effect of *H. kunzii* on *S. aureus* virulence genes expression

To look into the possibility of direct attenuation of *S. aureus* virulence by *H. kunzii*, the expression levels of two representative virulence genes (*hla* and *spa*) and the main regulatory gene *agr* (that influences the expression of numerous *S. aureus* virulence genes) were measured for the different *S. aureus* strains associated with the *H. kunzii* isolate H13 and compared to those of *S. aureus* alone (Figure 4).

We observed that *hla* gene which encodes for the α -hemolysin (representing one of the most important virulence factors) was significantly derepressed in *S. aureus* NSA739 associated with *H. kunzii* H13 (Median -0.277; 95%CI [-0.41 / -0.18]; $p < 0.01$). In the same way, *agr* was also significantly down regulated (Median -0.582; 95%CI [-0.36 / -0.81]; $p < 0.001$). *spa* gene which encodes the protein A (representing one of the most important colonizing factor) showed a significant overexpression in *S. aureus* NSA739 associated with *H. kunzii* H13 (Median 0.197; 95%CI [0.15-0.27]; $p < 0.001$). The same results were noted for all the *S. aureus* studied.

This data suggested that *H. kunzii* attenuated directly the virulence of *S. aureus* by a deregulation of virulence genes and the global regulator of this virulence.

Effect of sequential infections of *C. elegans*

To get a better understanding of the role of *H. kunzii* on *S. aureus* virulence, we evaluated the effect of sequential infections on nematodes. Firstly, we infected *C. elegans* with the different bacteria alone and in association during 12 h followed by a transfer of nematodes on OP50 strain. The results showed that *S. aureus* is clearly more virulent compared to *H. kunzii* ($P < 0.001$) and this virulence was not due to a constant reinfection since all the results were comparable to those obtained in the first experiments (Tables 2 and 3).

Secondly, we sequentially inoculated the different associated bacteria. We infected *C. elegans* with the *H. kunzii* strains alone during 12 h followed by a transfer of nematodes on the different *S. aureus* strains tested. We observed that the LT50s for this protocol were significantly reduced (LT50: 2.5-4.1 vs 4.1-5.7 days, respectively; $P < 0.001$). However, this impact was not clearly equivalent for the different combinations tested. Indeed, for the *H. kunzii* strain (H10) with different impact on the *S. aureus* virulence, the LT50 was similar to LT50 obtained for nematodes infected by NSA739 alone (LT50: 2.5 vs 2.8 days, respectively; P value, NS). For the *H. kunzii* strain (H13) with an impact on the virulence of all *S. aureus*, LT50 remained significantly reduced compared to nematodes infected with NSA739 alone (LT50: 4.1 vs 2.8 days, respectively; $P < 0.001$) (Table 2). Thus, the direct association of *H. kunzii* and *S. aureus* has an impact on the attenuation of *S. aureus* virulence. This effect is significantly reduced or aborted when the infection is sequential suggesting the necessity to simultaneously co-infect with both *H. kunzii* and *S. aureus* to attenuate the virulence of *S. aureus*. *H. kunzii* seems to act directly on *S. aureus* reducing its virulence and thus the host response (showed by the reduction of *cyp-37B1* and *dlec-7* expression previously).

Discussion

Social interactions involving parasites, protozoa and prokaryotes have been frequently described (Tourret *et al.*, 2011). Microbial co-occurrence networks indicate that bacterial species co-infect the same site of the human body and form microbial communities (Fernandez *et al.*, 2015). However documentations concerning interactions between non-virulent and pathogenic microorganisms are scarce particularly in DFU. In this article we show for the first time evidence of the modulation of *S. aureus* virulence when associated with a commensal bacterium, *H. kunzii*, frequently found associated in chronic wounds of the lower limbs (Vergne *et al.*, 2015).

Some studies described the interactions between *S. aureus* and other pathogens (Bourdon *et al.*, 2011; Tenover *et al.*, 1995; Lavigne *et al.*, 2013; Garsin *et al.*, 2001; Doumith *et al.* 2009). These interactions vary between bacteria: cooperation with *E. faecalis* and *Candida albicans* (Bourdon *et al.*, 2011; Tenover *et al.*, 1995; Lavigne *et al.*, 2013; Garsin *et al.*, 2001; Doumith *et al.* 2009). These interactions vary between bacteria: cooperation with *E. faecalis* and *Candida albicans*, competition with *Lactobacillus* sp. (Otto *et al.*, 2013), and *Streptococcus pneumoniae* (Garzoni *et al.*, 2007). *S. aureus* can also have both interactions (competition and cooperation) with the same pathogen depending of the disease and the conditions such as *Pseudomonas aeruginosa* (Tenover *et al.*, 1995; Lavigne *et al.*, 2013; Garsin *et al.*, 2011; Irazoqui *et al.*, 2008). If *P. aeruginosa* seems to never coaggregate with *S. aureus* in chronic wound ulcers (Livak and Schmittgen, 2001), these bacteria could share some siderophores to favour the growth of each other (Pfaffl, 2001). Moreover, our team has recently demonstrated the coexistence of two *S. aureus* population on DFU notably one with a very low virulence potential (Messad *et al.*, 2015). In this context, the study of the effect of *H. kunzii* is of particular interest. Although we confirmed that this microorganism has a low virulence potential in the nematode model (74% tested strains were non-virulent and 26% harboured a low-virulence profile), some studies have described that *H. kunzii* can also be an opportunistic pathogen (Lemaître *et al.*, 2008), notably in chronic wounds (Riegel and Lepargneur, 2003; Vergne *et al.*, 2015; Nair *et al.*, 2014; Baldan *et al.*, 2014). Its frequent association with *S. aureus* on DFU reinforced the need of a better understanding of the cooperation mechanisms between the two bacteria. Here, we observed that all the *H. kunzii* isolates associated with a virulent *S. aureus* strains (NSA739) clearly increased the lifespan of the *C. elegans* (LT50s: 4.1 to 5.7 vs 2.8 days, $P < 0.001$). This effect was confirmed when the nematodes were infected with *H. kunzii* and two other less virulent *S. aureus* strains (the reference strain Newman and a DFU colonizing strain NSA1385). To explain these results, two hypotheses could be made: i) *H. kunzii* modulated the immune response of *C. elegans* and help them to be more resistant or tolerant to *S. aureus*, ii) *H. kunzii* modulated directly the *S. aureus* virulence.

Previous experiments showed that primary infection with *S. aureus* can increase vulnerability of *C. elegans* and modify its tolerance to an opportunistic pathogen *Proteus mirabilis* (JebaMercy and Balamurugan, 2012). The sequential infections of nematodes provide the evidence that *H. kunzii* does not affect the tolerance of *C. elegans* to *S. aureus*. The fact that nematodes tolerate more *S. aureus* when they are mixed with *H. kunzii* could be due to a direct interaction between the two strains. *H. kunzii* may have a direct effect on *S. aureus* by interfering with the expression of *S. aureus* virulence genes. To confirm this hypothesis, we analysed the expression of main genes involved in nematode defense response after infection with *S. aureus*, *H. kunzii* and both. If *H. kunzii* strains did not modify these genes expression, *S. aureus* strains had a clear effect on the expression of *C. elegans* host defense genes particularly *hlh-30*, *cyp37*, *lys-5* and *sodh-1*, whatever the virulence of the strain. This observation is consistent with two studies showing that after 8 h of infection with *S. aureus*, *C. elegans* modified the production of defense genes (*clec-71*, *sodh-11*, *cyp-37B1*, *lys-5*) that have xenobiotic detoxification potential or antimicrobial activities, and then protect host by participating to host response (Visvikis *et al.*, 2014; Irazoqui *et al.*, 2010).

Also, our results show that *H. kunzii* does not modulate the immune response of *C. elegans* and the effect observed was due to a direct interaction between *H. kunzii* and *S. aureus* virulence. The downregulation of *hla* and *agr* expression in *S. aureus* co-cultured with *H. kunzii* sustained this hypothesis. In presence of *H. kunzii*, *S. aureus* could be in a 'colonizing' behaviour (as suggested by the overexpression of *spa* gene). Taken together, our work also confirms that *C. elegans* are not just a simple model to study pathogens' virulence. It is an entire organism that can establish immune mechanism to fight against infection and depending to the pathogen agent, can stimulate host defense genes (Visvikis *et al.*, 2014; Irazoqui *et al.*, 2010; Hoffman *et al.*, 2006). Nematodes use some metabolic pathway of defense and express some genes that share similarities and/or homologies with those expressed during vertebrate and human infection (Irazoqui *et al.*, 2010). Further investigations need to be carried out in order to define by which mechanism(s) *Helcococcus* may alter *S. aureus* virulence.

To the best of our knowledge this is the first description of a virulence-modulating bacterial interaction between a non-virulent bacterial species and a naturally occurring pathogenic strain. This virulence attenuation was independent to host defense mechanisms. We believe that this observation provides a new insight into *S. aureus* virulence. The possibility that a non-virulent commensal strain impacts the virulence of *S. aureus* is of great interest, considering the numbers of commensal bacteria contained in DFU (Gardner *et al.*, 2013). Our results obtained in a model organism emphasize the importance of studying the connections between pathogenic species and the endogenous microbiota. If pathogenic bacteria are well characterized in infection, they cannot be reduced to a single organism infecting host. All the bacteria participate to the chronicity of the wound at different levels and their virulence modulation has to be investigated to a best management of the wounds. This could also represent new ways to fight infections.

Funding

This work was supported by INSERM.

Acknowledgments

Fer-15 nematodes were provided by the Caenorhabditis Genetics Center, a foundation of the NIH National Center for Research Resources (NCRR). We thank Anne Keriell for her help in this study and Mariella Lomma for her editing assistance.

Author Contributions

JPL, CDR, OV, EL and AS conceived and designed the experiments. CNE, OV, MFG, VC and CDR performed the experiments. MFG, AV, VC, AL provided the *Helcococcus* strains. CNE, OV, MFG, EL, AS, JPL, and CDR analyzed the data. CNE, JPL and CDR wrote the paper. OV, MFG, AV, VC, AL, EL, and AS reviewed and edited the manuscript.

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Figure legends

Figure 1. In vivo kinetics of killing of *C. elegans* infected by *S. aureus* NSA739, *H. kunzii* H13 and co-infected by the two strains. OP50 represents the survival curve for worms fed on non-pathogenic *E. coli*. In all cases, worms were grown on NGM plates at 25°C and ≈ 30 Fer-15 were used in each test. The curves are representative of at least three independent trials for each group of strains.

Figure 2. Evaluation of feeding behaviour by measuring bacterial content of *C. elegans*. Each graph represents the median of CFU count found in each species alone (the *H. kunzii* H13, the *S. aureus* NSA739 and the *E. coli* OP50) or in co-infection (*H. kunzii* H13 + *S. aureus* NSA739). Three replicates were performed for each strain alone or in association. Differences in CFU rates were tested by a Pearson normality test. These results are representative of the data obtained for all the strains evaluated in this study. NS: not significant

Figure 3. *C. elegans* L4 host response to *S. aureus* and *H. kunzii* infections alone or in association at 12 hours post infection. The figure represents the expression of six main genes involved in *C. elegans* immune response: *cyp-37B1* (A), *hlh-30* (B), *lys-5* (C), *sodh-1* (D), *clec-7* (E), *lgg-1* (F). Data are expressed as the mean $\pm$ sd of three biological replicates of qRT-PCR results. All Ct values are normalized against the housekeeping gene *snb-1*. Data analysis was performed with the Pfaffl method (53). The P value represents the comparison between genes expression found in *H. kunzii* strains alone and the other combination (*S. aureus* alone, co-infection *H. kunzii*+*S. aureus*) *, *P* values < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Figure 4. Relative mRNA expression level of genes implicated in virulence (*hla*, *spa*) and virulence regulation (*agr*) for *S. aureus* NSA739 co-infected with *H. kunzii* H13.

The log-transformed averages of relative fold change of *S. aureus*+*H. kunzii* co-infection compared to *S. aureus* alone are presented. The error bars represent the standard deviation from three different RNA preparations. Significant differences from *S. aureus* co-infected with *H. kunzii* using Dunnett's test are indicated by ** (*p* < 0.01) and *** (*p* < 0.001).

Table 1 50% Lethal Time of *Caenorhabditis elegans* infected with the different *S. aureus* and *H. kunzii* strains and evaluation of feeding behaviour by measuring the pathogen avoidance. The results are representative of at least five independent assays for each group of strains. *P*: Pairwise comparison between LT50s using a log rank test. NS: not significant. LT50: 50% Lethal Time.

Strain	Characteristics of the strain (References)	LT50 in days (IC95% inf-sup)	Occupancy test after 16h (%)	<i>P</i>	OP50 vs others	<i>P</i>	NSA739 vs others	<i>P</i>	NSA1385 vs others	<i>P</i>
NSA1385	<i>S. aureus</i> , clinical, colonizing (28)	4.7 (4.5-4.8)	98 ±2	<0.001	<0.001	<0.001	-	NS		
NSA739	<i>S. aureus</i> , clinical, infecting (28)	2.8 (2.4-3.0)	96 ±4	<0.001	<0.001	-	<0.001	<0.001		
Newman	<i>S. aureus</i> , reference	4.3 (4.0-4.6)	95 ±4	<0.001	<0.001	<0.001	NS	-		
H1	<i>H. kunzii</i> , clinical, colonizing (23)	7.2 (6.4-8.0)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		
H2	<i>H. kunzii</i> , clinical, colonizing (23)	7.2 (6.4-8.0)	92 ±5	NS	<0.001	<0.001	<0.001	<0.001		
H3	<i>H. kunzii</i> , clinical, colonizing (23)	7.0 (6.4-7.6)	95 ±3	NS	<0.001	<0.001	<0.001	<0.001		
H4	<i>H. kunzii</i> , clinical, colonizing (23)	7.5 (6.8-8.2)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		
H6	<i>H. kunzii</i> , clinical, colonizing (23)	6.1 (5.9-6.5)	97 ±3	NS	<0.001	<0.001	<0.001	<0.001		
H7	<i>H. kunzii</i> , clinical, colonizing (23)	5.7 (5.5-5.9)	98 ±3	<0.001	<0.001	<0.001	<0.001	<0.001		
H8	<i>H. kunzii</i> , clinical, colonizing (23)	6.8 (6.4-7.2)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		
H9	<i>H. kunzii</i> , clinical, colonizing (23)	8.0 (7.5-8.5)	97 ±3	NS	<0.001	<0.001	<0.001	<0.001		
H10	<i>H. kunzii</i> , clinical, colonizing (23)	5.5 (4.6-6.4)	96 ±4	<0.001	<0.001	<0.001	<0.001	<0.001		
H11	<i>H. kunzii</i> , clinical, colonizing (23)	7.5 (7.3-7.7)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		
H12	<i>H. kunzii</i> , clinical, colonizing (23)	6.2 (5.8-6.6)	99 ±1	NS	<0.001	<0.001	<0.001	<0.001		
H13	<i>H. kunzii</i> , clinical, colonizing (23)	6.3 (5.8-6.8)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		
H14	<i>H. kunzii</i> , clinical, colonizing (23)	6.7 (5.9-7.5)	92 ±3	NS	<0.001	<0.001	<0.001	<0.001		
H16	<i>H. kunzii</i> , clinical, colonizing (23)	6.5 (6.2-6.7)	100 ±0	NS	<0.001	<0.001	<0.001	<0.001		

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H17a	<i>H. kunzii</i> , clinical, colonizing (23)	5.6 (5.0-6.2)	94 ±4	<0.001	<0.001	<0.001	<0.001
H17b	<i>H. kunzii</i> , clinical, colonizing (23)	7.4 (7.2-7.6)	100 ±0	NS	<0.001	<0.001	<0.001
H18	<i>H. kunzii</i> , clinical, colonizing (23)	7.1 (6.7-7.5)	100 ±0	NS	<0.001	<0.001	<0.001
H19	<i>H. kunzii</i> , clinical, colonizing (23)	6.6 (6.1-7.1)	100 ±0	NS	<0.001	<0.001	<0.001
H20	<i>H. kunzii</i> , clinical, colonizing (23)	5.8 (5.3-6.1)	96 ±2	<0.001	<0.001	<0.001	<0.001
H21	<i>H. kunzii</i> , clinical, colonizing (23)	6.0 (5.4-6.6)	100 ±0	<0.001	<0.001	<0.001	<0.001
H22a	<i>H. kunzii</i> , clinical, colonizing (23)	7.4 (7.0-7.9)	91 ±3	NS	<0.001	<0.001	<0.001
H22b	<i>H. kunzii</i> , clinical, colonizing (23)	5.6 (5.0-6.2)	100 ±0	<0.001	<0.001	<0.001	<0.001
H23	<i>H. kunzii</i> , clinical, colonizing (23)	6.5 (6.1-7.0)	95 ±4	NS	<0.001	<0.001	<0.001
OP50	<i>E. coli</i> , control strain	7.1 (6.6-7.7)	100 ±0	-	<0.001	<0.001	<0.001



Table 2 50% Lethal Time of *Caenorhabditis elegans* co-infected with a virulent *S. aureus* strain (NSA739) and *H. kunzii* strains and evaluation of feeding behaviour by measuring the pathogen avoidance. The results are representative of at least four independent assays for each group of strains. *P*: Pairwise comparison between LT50s using a log rank test. NS: not significant. LT50: 50% Lethal Time. Infection of nematodes with H10 or H13 followed by transfer on ψ OP50 or * *S. aureus* 12 h after.

Strain	LT50 in days (IC95% inf-sup)	Occupancy test after 16h (%)	<i>P</i> OP50 vs others	<i>P</i> NSA739 vs others	<i>P</i> H10 vs others	<i>P</i> H13 vs others
NSA739	2.8 (2.4-3.0)	96 ±4	<0.001	-	<0.001	<0.001
H10	5.5 (4.6-6.4)	96 ±4	<0.001	<0.001	-	NS
H13	6.2 (5.8-6.6)	100 ±0	NS	<0.001	NS	-
H1 + NSA739	5.0 (4.9-5.1)	100 ±0	<0.001	<0.001	NS	<0.001
H2 + NSA739	4.8 (4.5-5.0)	95 ±5	<0.001	<0.001	NS	<0.001
H3 + NSA739	4.7 (4.6-4.8)	92 ±4	<0.001	<0.001	NS	<0.001
H4+ NSA739	5.3 (5.1-5.7)	100 ±0	<0.001	<0.001	NS	NS
H6+ NSA739	4.4 (4.3-4.6)	100 ±0	<0.001	<0.001	<0.001	<0.001
H7+ NSA739	4.7 (4.6-4.8)	94 ±6	<0.001	<0.001	NS	<0.001
H8+ NSA739	4.3 (4.2-4.4)	95 ±3	<0.001	<0.001	<0.001	<0.001
H9+NSA739	4.8 (4.7-4.9)	100 ±0	<0.001	<0.001	NS	<0.001
H10+ NSA739	4.1 (4.0-4.3)	92 ±5	<0.001	<0.001	<0.001	<0.001
H11+ NSA739	5.2 (5.1-5.4)	90 ±5	<0.001	<0.001	NS	NS
H12+ NSA739	4.4 (4.3-4.5)	100 ±0	<0.001	<0.001	<0.001	<0.001
H13+ NSA739	5.7 (5.3-5.9)	100 ±0	<0.001	<0.001	<0.001	NS
H14+ NSA739	4.7 (4.6-4.8)	97 ±3	<0.001	<0.001	NS	<0.001
H16+ NSA739	5.1 (4.9-5.5)	98 ±3	<0.001	<0.001	NS	<0.001

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H17a+ NSA739	4.5 (4.3-4.6)	100 ±0	<0.001	<0.001	NS	<0.001
H17b+ NSA739	5.3 (5.0-5.5)	93 ±5	<0.001	<0.001	NS	NS
H18+ NSA739	5.2 (5.0-5.4)	100 ±0	<0.001	<0.001	NS	NS
H19+NSA739	4.8 (4.7-4.9)	100 ±0	<0.001	<0.001	NS	<0.001
H20+ NSA739	4.6 (4.5-4.8)	91 ±5	<0.001	<0.001	NS	<0.001
H21+ NSA739	4.7 (4.6-4.9)	98 ±2	<0.001	<0.001	NS	<0.001
H22a+ NSA739	4.7 (4.6-4.8)	94 ±4	<0.001	<0.001	NS	<0.001
H22b+ NSA739	5.0 (4.8-5.3)	100 ±0	<0.001	<0.001	NS	<0.001
H23+ NSA739	5.1 (4.9-5.3)	98 ±2	<0.001	<0.001	NS	<0.001
H10> +OP50 ^ψ	6.6 (6.2-6.8)	100 ±0	NS	<0.001	<0.001	NS
H13> +OP50 ^ψ	6.2 (5.8-6.6)	97 ±3	NS	<0.001	NS	NS
NSA739> +OP50 ^ψ	2.8 (2.4-3.0)	96 ±4	<0.001	NS	<0.001	<0.001
NSA1385> +OP50 ^ψ	4.4 (4.0-5.1)	100 ±0	<0.001	<0.001	<0.001	<0.001
Newman> +OP50 ^ψ	4.0 (3.5-4.3)	94 ±4	<0.001	<0.001	<0.001	<0.001
H10> +NSA739 *	2.5 (2.4-2.7)	100 ±0	<0.001	NS	<0.001	<0.001
H13> +NSA739 *	4.1 (3.7-4.4)	100 ±0	<0.001	<0.001	<0.001	<0.001
OP50	7.1 (6.6-7.7)	100 ±0	-	<0.001	<0.001	<0.001



Table 3 50% Lethal Time of *Caenorhabditis elegans* co-infected with *S. aureus* strains and *H. kunzii* strains and evaluation of feeding behaviour by measuring the pathogen avoidance. The results are representative of at least four independent assays for each group of strains. *P*: Pairwise comparison between LT50s using a log rank test. NS: not significant. LT50: 50% Lethal Time. Infection of nematodes with H10 or H13 followed by transfer on * *S. aureus* 12 h after.

Strain	LT50 in days (IC95% inf-sup)	Occupancy test after 16h (%)	<i>P</i> OP50 vs others	<i>P</i> NSA1385 vs others	<i>P</i> Newman vs others	<i>P</i> H10 vs others	<i>P</i> H13 vs others
NSA1385	4.7 (4.5–4.8)	98 ±2	<0.001	-	NS	NS	<0.001
Newman	4.3 (4.0–4.6)	95 ±4	<0.001	NS	-	<0.001	<0.001
H10	5.5 (4.6–6.4)	96 ±4	<0.001	NS	<0.001	-	NS
H13	6.2 (5.8–6.6)	100 ±0	NS	<0.001	<0.001	NS	-
H1 + NSA1385	5.0 (4.8–5.3)	96 ±4	<0.001	NS	)	NS	<0.001
H1 + Newman	3.3 (3.1–3.4)	96 ±4	<0.001	<0.001	NS	<0.001	<0.001
H2 + NSA1385	5.3 (5.1–5.5)	100 ±0	<0.001	NS	-	NS	NS
H2 + Newman	5.2 (5.0–5.7)	95 ±4	<0.001	NS	NS	NS	<0.001
H3 + NSA1385	4.7 (4.5–4.8)	100 ±0	<0.001	NS	-	NS	<0.001
H3 + Newman	3.8 (3.6–3.9)	92 ±3	<0.001	NS	NS	<0.001	<0.001
H4+ NSA1385	5.9 (5.8–6.1)	100 ±0	<0.001	<0.001	-	NS	NS
H4 + Newman	4.8 (4.4–5.2)	100 ±0	<0.001	NS	NS	NS	<0.001
H6+ NSA1385	5.8 (5.6–6.0)	100 ±0	<0.001	<0.001	-	NS	NS
H6 + Newman	4.0 (3.8–4.2)	91 ±4	<0.001	NS	NS	<0.001	<0.001
H7+ NSA1385	5.6 (5.3–5.8)	100 ±0	<0.001	NS	-	NS	NS
H7 + Newman	4.4 (4.2–4.6)	100 ±0	<0.001	NS	NS	<0.001	<0.001
H8+ NSA1385	5.9 (5.7–6.2)	92 ±3	<0.001	<0.001	-	NS	NS

Résultats

H8 + Newman	3.6 (3.5-3.8)	100 ±0	<0.001	<0.001	NS	<0.001	<0.001
H9+ NSA1385	5.0 (4.9-5.1)	100 ±0	<0.001	NS	-	NS	<0.001
H9 + Newman	6.1 (5.9-6.2)	95 ±4	<0.001	<0.001	<0.001	NS	NS
H10+ NSA1385	4.0 (3.9-4.2)	100 ±0	<0.001	NS	-	<0.001	<0.001
H10 + Newman	3.6 (3.5-3.7)	90 ±5	<0.001	<0.001	NS	<0.001	<0.001
H11+ NSA1385	5.3 (5.0-5.5)	100 ±0	<0.001	NS	-	NS	NS
H11 + Newman	4.0 (3.9-4.2)	91 ±4	<0.001	NS	NS	<0.001	<0.001
H12+ NSA1385	5.0 (4.9-5.3)	97 ±3	<0.001	NS	-	NS	<0.001
H12 + Newman	4.2 (4.1-4.3)	90 ±5	<0.001	NS	NS	<0.001	<0.001
H13+ NSA1385	5.8 (5.7-5.9)	100 ±0	<0.001	<0.001	-	NS	NS
H13 + Newman	6.3 (6.2-6.4)	90 ±5	NS	<0.001	<0.001	NS	NS
H14+ NSA1385	5.1 (4.9-5.4)	97 ±3	<0.001	NS	-	NS	<0.001
H14 + Newman	5.1 (4.9-5.2)	95 ±4	<0.001	NS	NS	NS	<0.001
H16+ NSA1385	6.3 (6.1-6.4)	90 ±5	NS	<0.001	-	NS	NS
H16 + Newman	4.6 (4.2-5.0)	100 ±0	<0.001	NS	NS	NS	<0.001
H17a+ NSA1385	5.2 (5.0-5.4)	100 ±0	<0.001	NS	-	NS	<0.001
H17a + Newman	4.1 (3.9-4.4)	100 ±0	<0.001	NS	NS	<0.001	<0.001
H17b+ NSA1385	5.7 (5.3-6.0)	92 ±3	<0.001	<0.001	-	NS	NS
H17b + Newman	5.4 (5.2-5.6)	98 ±2	<0.001	NS	<0.001	NS	NS
H18+ NSA1385	5.7 (5.3-6.1)	100 ±0	<0.001	<0.001	-	NS	NS
H18 + Newman	5.9 (5.6-6.2)	97 ±3	<0.001	<0.001	<0.001	NS	NS
H19+ NSA1385	4.8 (4.6-5.0)	90 ±5	<0.001	NS	-	NS	<0.001
H19 + Newman	3.8 (3.7-3.9)	100 ±0	<0.001	NS	NS	<0.001	<0.001



Résultats							
H20+ NSA1385	5.2 (5.1-5.3)	92 ±3	<0.001	NS	-	NS	<0.001
H20 + Newman	4.5 (4.3-4.6)	94 ±6	<0.001	NS	NS	<0.001	<0.001
H21+ NSA1385	4.6 (4.5-4.8)	100 ±0	<0.001	NS	-	NS	<0.001
H21 + Newman	4.7 (4.3-5.0)	94 ±6	<0.001	NS	NS	NS	<0.001
H22a+ NSA1385	5.7 (5.6-5.8)	95 ±4	<0.001	<0.001	-	NS	NS
H22a + Newman	6.0 (5.9-6.1)	92 ±3	<0.001	<0.001	<0.001	NS	NS
H22b+ NSA1385	6.1 (5.8-6.4)	100 ±0	NS	<0.001	-	NS	NS
H22b + Newman	5.9 (5.4-6.3)	98 ±2	<0.001	<0.001	<0.001	NS	NS
H23+ NSA1385	6.0 (5.9-6.1)	100 ±0	<0.001	<0.001	-	NS	NS
H23 + Newman	7.1 (6.8-7.4)	92 ±3	NS	<0.001	<0.001	<0.001	NS
H10> + NSA1385 *	4.9 (4.7-5.1)	100 ±0	<0.001	NS	-	NS	<0.001
H10> + Newman *	4.6 (4.3-4.8)	95 ±4	<0.001	NS	NS	NS	<0.001
H13> + NSA1385 *	5.2 (5.0-5.3)	100 ±0	<0.001	NS	-	NS	<0.001
H13> + Newman *	5.5 (5.2-5.7)	92 ±3	<0.001	NS	<0.001	NS	NS



Table 4 Primers used in the study

Primer use and target function	Target region	Primer name	Oligonucleotide sequence	Tm (°C)	Reference
Host qRT-PCR					
Transcriptional factor for host defense	<i>hlh-30</i>	hlh-30 F	5'-CGGGGCTGGCTCAGGACACTC-3'	65.5	24
		hlh-30 R	5'-GGCGCCCGAACTTGAGACGAC-3'	63.5	
Antimicrobial function	<i>lys-5</i>	lys-5 F	5'-CGGGGCTGGCTCAGGACACTC-3'	54.7	24
		lys-5 R	5'-GGCGCCCGAACTTGAGACGAC-3'	53.2	
	<i>clec-7</i>	clec-7 F	5'-TTTATGGGACGATTCCGACGG-3'	57.3	24
		clec-7 R	5'-GTCAATGCACCTTGACGGA-3'	57.3	
Cytoprotection	<i>cyp-37B1</i>	cyp-37B1 F	5'-GAATGTATCCGTCAGTGCCA-3'	57.3	25
		cyp-37B1 R	5'-TCGGACTCCTTTTGGGAAGA-3'	57.3	
Detoxification	<i>sodh-1</i>	sodh-1 F	5'-CTGGATGGCAACTTGGAGACAAAGC-3'	64.6	51
		sodh-1 R	5'-GGTGGCAGAGTGGCTCGTGG-3'	65.5	
Autophagy	<i>lgg-1</i>	lgg-1 F	5'- ACCATGACCACAATGGGACAAC C-3'	62.7	24
			5'-ACACTTTCGTCACTGTAGGCGATG-3'	62.7	

S. aureus qRT-PCR

© hemolysin	<i>hla</i>	Hla-F	5'-TCCAGTGCAATTGGTAGTCA-3'	55.3	48
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Protein A	<i>spa</i>	Hla-R	5'-GGCTCTATGAAAGCAGCAGA-3'	57.3	
		Spa-F	5'-TATGCCCTAACTTAAATGCTG-3'	51.1	48
		Spa-R	5'-TTGGAGCTTGAGAGTCATT-3'	53.2	
Accessory gene regulator	<i>agrA</i>	F_agrA_34	5'-		
			CAAAAGAGAAAACA TGGTACCATTTATTAA	58.2	49
		R_agrA_135	5'- CTCAAGCACCTCATAAGGATTATCAG-3'	61.6	



Figure 1.

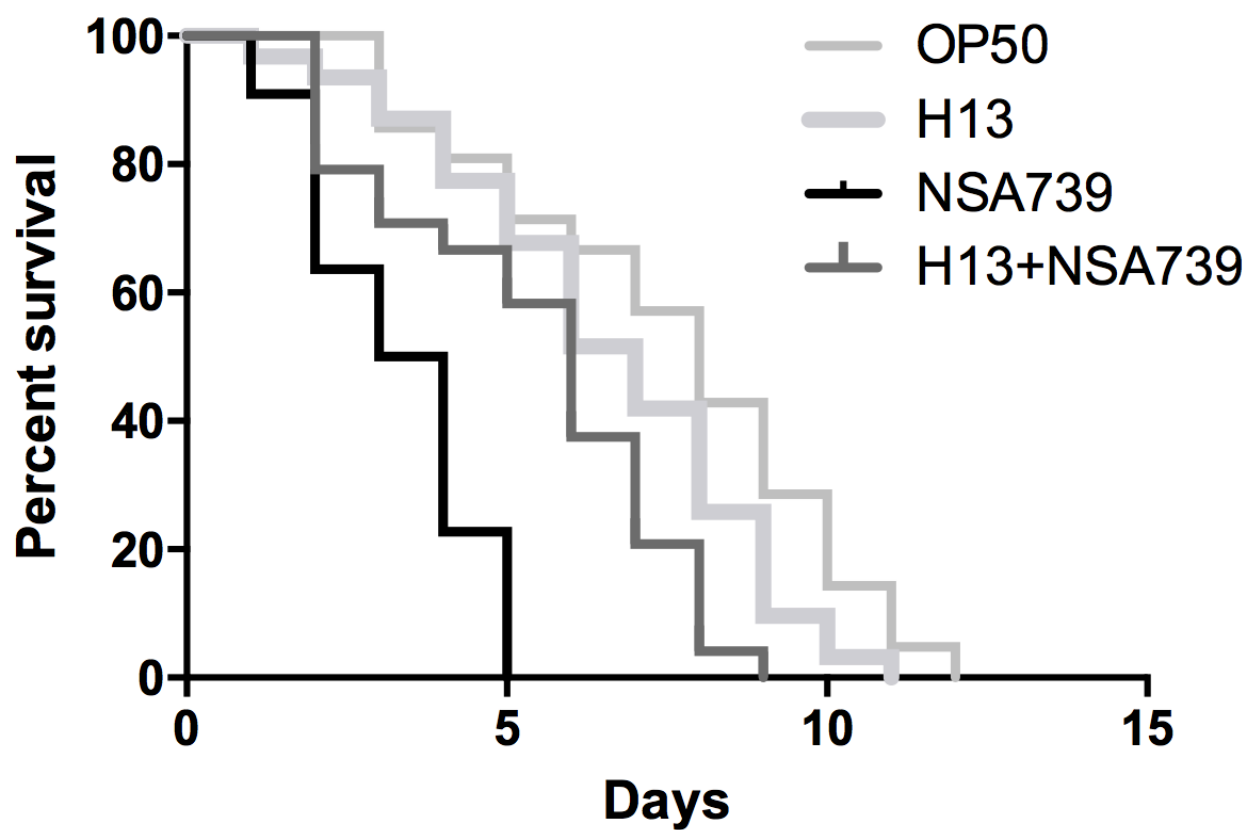


Figure 2.

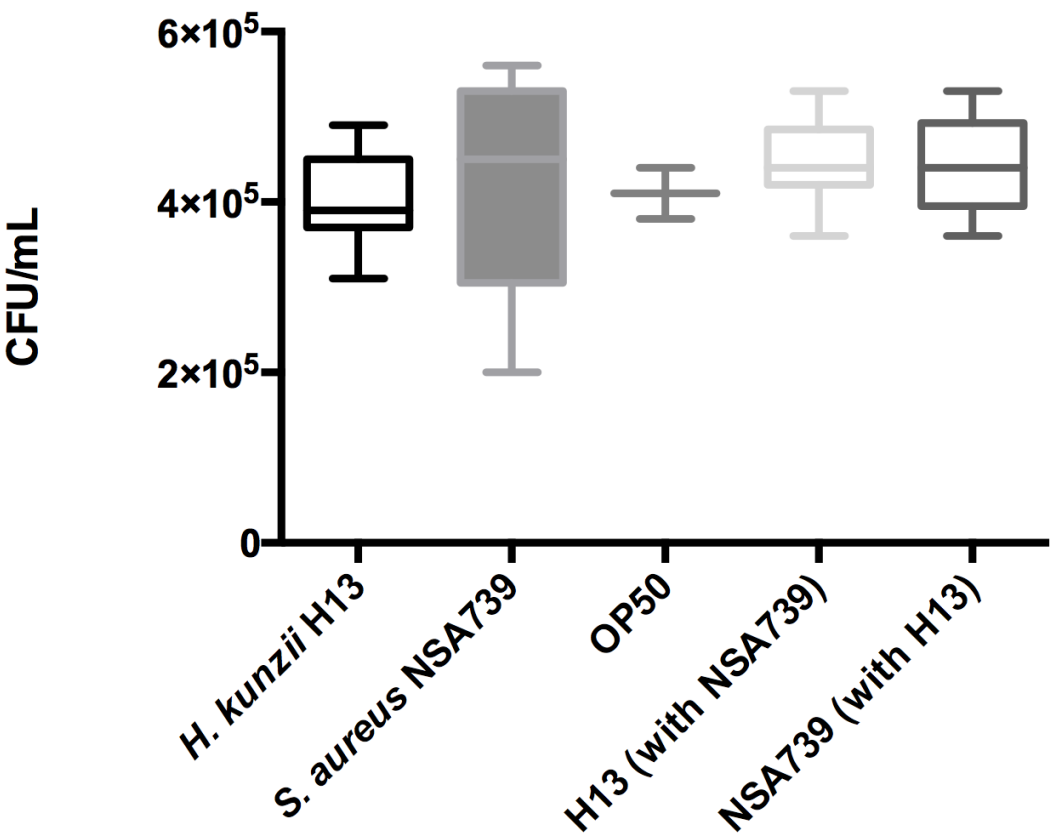


Figure 3.

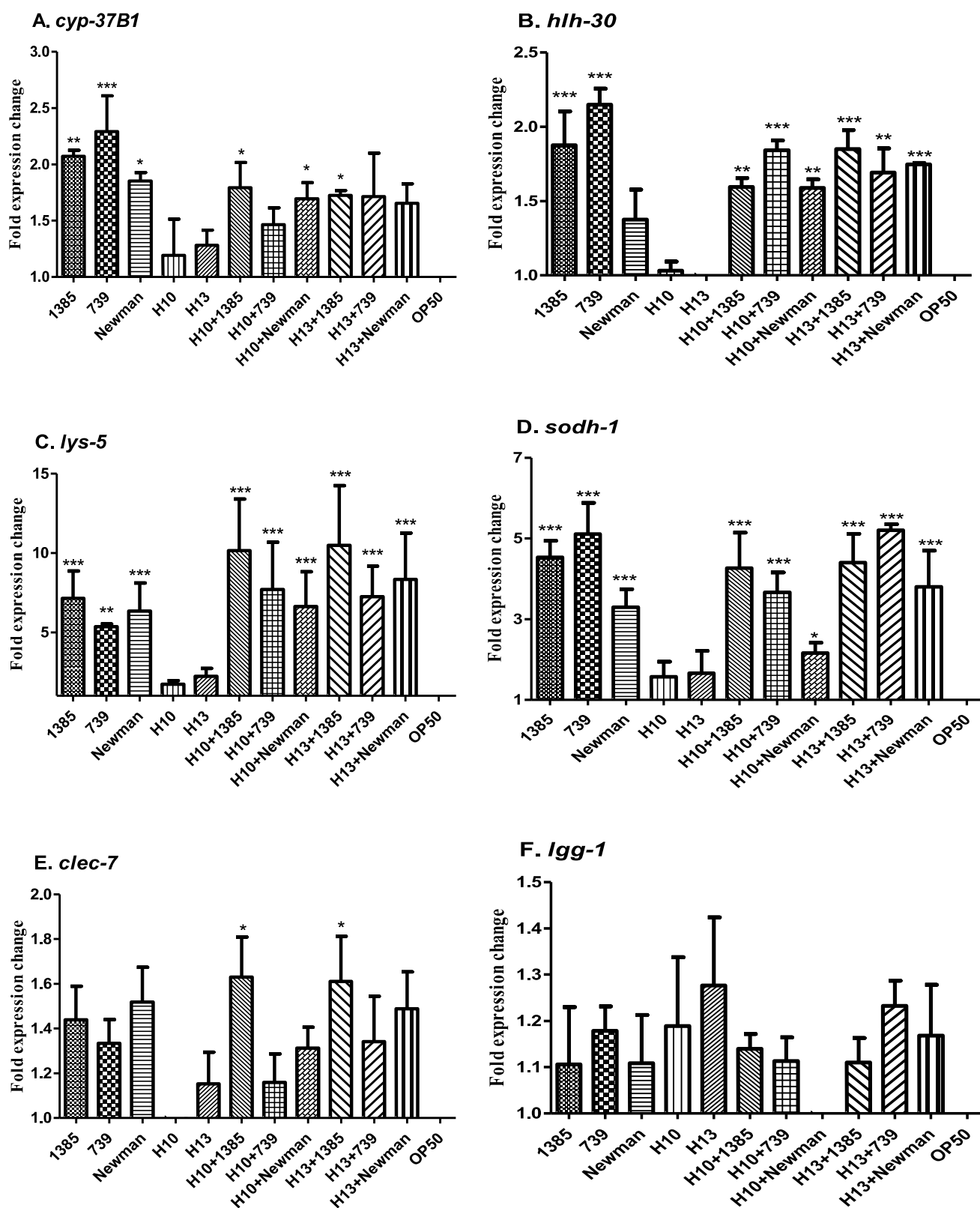
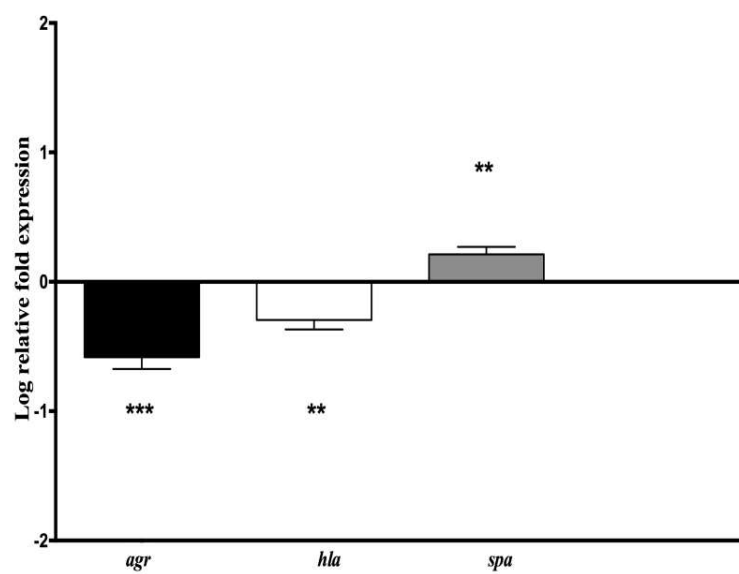




Figure 4.



DISCUSSION - PERSPECTIVES

Le diabète sucré est un problème mondial. L'une de ses complications principales est l'apparition de plaies situées au niveau du pied pouvant évoluer vers l'amputation des membres inférieurs chez ces patients. Plusieurs études ont montré que *S. aureus* était la principale bactérie fréquemment isolée lors de ces infections. Ce cocci à Gram positif aéro-anaérobie facultatif est d'une grande importance clinique car il est responsable de nombreuses infections en particulier au niveau des pieds pouvant aller de plaies superficielles à des infections sévères (choc septique).

Dans le but de mieux comprendre la virulence des bactéries présentes au niveau des plaies du pied chez le diabétique, d'améliorer la prise en charge des patients diabétiques et de développer de nouvelles stratégies thérapeutiques, nous nous sommes posés plusieurs questions :

- Les souches colonisantes de *S. aureus* isolées de plaies du pied conservent-elles constamment le phage ROSA-like, signature de leur état de colonisant ou peuvent-elles le perdre facilement et devenir de réelles bactéries infectantes ?
- Quel impact a le phage ROSA-like sur le métabolisme des souches colonisantes ?
- Est-ce que les facteurs environnementaux rencontrés par les bactéries au niveau des plaies influencent la virulence des souches de *S. aureus* isolées de plaies du pied ?
- Enfin lorsque *S. aureus* n'est pas seul mais associé à une bactérie commensale comme *H. kunzii*, fréquemment isolé en association avec *S. aureus*, existe-t-il des modifications de la virulence de *S. aureus* ?

Dans un premier temps, notre travail a porté sur une souche de *S. aureus* isolée de plaie du pied chez le diabétique. Cette souche isolée au laboratoire INSERM de Nîmes a pour particularité d'avoir un très faible potentiel de virulence du fait de la présence du phage ROSA-like. Le travail précédent de l'équipe avait indiqué que l'absence du phage rétablissait la virulence de la souche. Il était donc important de vérifier la stabilité du phage en particulier dans des conditions environnementales de stress rencontrées lors de plaies du pied chez le diabétique. Après avoir cultivé ces bactéries colonisantes dans plusieurs conditions stringentes (hyperglycémie, anaérobiose, milieu minium, antibiotiques, ultraviolet...), nous avons vu que le phage ROSA-like était extrêmement stable dans cette souche et ne pouvait pas être excisé facilement. Ce résultat est capital car il signifie que l'identification en clinique d'une souche

colonisante possédant le phage ROSA-like (donc ayant un très faible potentiel de virulence) induit l'inutilité de mettre en place un traitement antibiotique.

Par ailleurs, la localisation de ce phage près du locus *isd*, locus intervenant dans le métabolisme du fer chez *S. aureus* nous a intrigué nous conduisant à évaluer l'impact de ce phage dans le métabolisme de la souche colonisante, particulièrement dans le métabolisme du fer. En effet, le fer a été décrit comme étant un élément essentiel pour les bactéries car il intervient dans plusieurs métabolismes cellulaires⁷⁵. L'absence de fer est bactériostatique pour la bactérie voir bactéricide si cette absence est prolongée. C'est un cofacteur pour de nombreuses autres métalloprotéines³⁴ et pour les enzymes de défense contre le stress oxydatif⁷⁶ et c'est un élément nutritif limitant la croissance bactérienne³⁴. Cependant, à l'état naturel, le fer ne se trouve pas sous forme libre dans l'organisme mais sous forme lié en particulier à des protéines (comme par exemple l'hémoglobine). En absence de fer, la bactérie doit développer obligatoirement des mécanismes d'acquisition du fer. *Staphylococcus aureus* utilise deux voies, soit directement par les protéines Isd présentes à sa surface, soit indirectement par la production de sidérophores. Ces mécanismes sont sous la dépendance d'un régulateur global Fur.

Dans ce travail, nous avons donc montré par les analyses transcriptomiques que la présence du phage ROSA-like affectait complètement la capacité de *S. aureus* à assimiler le fer du fait d'une dérégulation du gène *fur* qui s'avère être continuellement exprimé même en l'absence de fer. Cette absence de levée de la répression de ce gène provoque la non expression des gènes codant pour les Isd et l'absence de production de sidérophores. Ces résultats sont en adéquation avec ce qui a déjà été démontré sur le rôle et le mécanisme d'action de Fur: Fur est un répresseur transcriptionnel des gènes impliqués dans l'acquisition du fer en se fixant sur les séquences consensus appelées Fur box. Cette répression s'effectue quand *S. aureus* se trouve dans un environnement riche en fer^{34,35,43}. Cependant, dans un environnement appauvri en fer, *fur* est réprimé entraînant l'augmentation de l'expression des gènes intervenant dans l'acquisition du fer (*isd* et gènes codant pour les sidérophores)³⁶. La dérégulation du gène *fur* est la conséquence de la présence du phage ROSA-like puisque l'absence de ce phage rétablit l'ensemble du mécanisme d'acquisition du fer. Durant ma thèse, j'ai travaillé sur l'identification du/des facteurs produit(s) par le phage et pouvant déréguler *fur*. Quatre gènes candidats ont été identifiés : *orf13*, *orf22*, *orf35*, *ssb* gènes sélectionnés puisqu'ils étaient surexprimés dans la souche colonisante par rapport à la souche Newman en présence et en absence de fer. Nous

avons alors essayé de générer des mutants pour chacun de ces gènes afin d'évaluer celui qui jouerait un rôle dans la dérégulation de *fur*. La technique utilisée pour générer les mutants consistait à amplifier la région en aval et celle en amont du gène d'intérêt et à les cloner dans un plasmid puC29 conservé dans une souche d'*Escherichia coli*. Par la suite ces fragments devaient être digérés, ligués et clonés dans un autre plasmid (PMAD) utilisé pour les bactéries complexes¹¹⁰. Ce dernier par électroporation devait être incorporé dans une souche de *S. aureus* RN4220. La méthode de transduction de phage devait nous servir à transformer la souche colonisante qui par double crossing-over devait subir la délétion de la région d'intérêt. Malgré plusieurs tentatives, aucun des mutants espérés n'a pu être généré ne permettant pas d'identifier le/les gène(s) impliqué(s) dans cette dérégulation.

Pour compléter l'étude du métabolisme de cette souche et mieux comprendre son rôle, en collaboration avec l'équipe du Professeur Schrenzel et avec l'aide de Patrice François à Genève, nous avons réalisé une étude transcriptionnelle en utilisant un microarray spécifique de la souche colonisante. En l'absence de fer, cette souche présentait de nombreuses particularités en comparaison avec la souche de référence Newman (qui a la particularité de posséder un phage situé exactement au même endroit que le phage ROSA-like). Ainsi, la souche colonisante présentait une sous expression importante des gènes codant pour l'adhésion et appartenant à la famille des MSCRAMM. Des expériences d'adhésion sur les cellules humaines de kératinocytes nous ont confirmé que la souche colonisante adhérait significativement moins que les autres souches dans un milieu pauvre en fer. La première étape de la pathogénicité de *S. aureus* étant l'adhésion^{111,112}, ces résultats pourraient expliquer pourquoi cette souche colonisante n'est pas virulente et n'est qu'exceptionnellement isolée en pathologie humaine (puisque'il est déconseillé de prélever des plaies colonisées).

De plus, les gènes codant pour les facteurs intervenant dans le métabolisme des pyrimidines, importants pour la synthèse d'ADN, la réparation et la réplication¹¹³ ainsi que les gènes codant pour les facteurs intervenant dans le métabolisme du nitrogène, nécessaire à la synthèse d'ATP, étaient inhibés dans cette souche. La coagulase et la gamma hémolysine étaient également sous exprimées dans cette souche en milieu pauvre en fer. Tous ces résultats montrent toutes les difficultés rencontrées par cette bactérie à s'adapter à ces conditions stringentes.

Toutefois, nous avons observé que cette souche réussissait tout de même à se maintenir en vie dans les conditions pauvres en fer. Selon Hall et Ji en 2013¹¹⁴, l'analyse métabolique du *S. aureus* dans des milieux complexes indiquent que *S. aureus* consomme premièrement le glucose, l'arginine et la thréonine. Les résultats obtenus par microarray ont confirmé ces

données puisque la souche colonisante activait les voies du fructose, de l'arginine, et des acides gras ainsi que le cycle de l'urée conduisant ainsi à l'activation de la glycolyse et la glycogénèse, permettant la production d'énergie. De plus, l'ABC transporteur *opp* était surexprimé par cette souche permettant l'acquisition du nickel et du cobalt, deux éléments importants pour la survie bactérienne ^{115,116}.

Ce travail nous a donc ainsi permis de comprendre que le phage ROSA-like était bien stable et inhibait la virulence des souches colonisantes principalement en bloquant les mécanismes de captation de fer nécessaires pour l'établissement de sa pathogénicité. Néanmoins cette souche réussit à se maintenir en vie par l'activation d'autres voies. Ceci nous permet donc de mieux comprendre ce qui se passe dans le pied diabétique et l'absence de cette souche en pathologie. Plusieurs questions restent à élucider : quel(s) facteur(s) du phage joue(nt) ce rôle de dérégulateur de *fur* ?, est-ce que les souches colonisantes font le lit des souches infectantes ? existe-t-il une niche physiologique de ces souches dans l'organisme ou l'environnement ?. Ces travaux ouvrent aussi la question de l'utilisation du phage comme moyen thérapeutique pour les infections à *S. aureus*. Des études de transduction de phage pourraient être mises en place pour voir dans quelle mesure ce phage pourrait être s'insérer dans les souches infectantes dans le but d'inhiber leur virulence.

Au sein des plaies du pied chez le diabétique, les souches bactériennes sont soumises à des conditions environnementales très particulières du fait même de la physiopathologie de cette lésion (artériopathie, neuropathie, cloisonnement du pied, diabète déséquilibré...). Ces conditions inévitablement doivent avoir un retentissement sur la virulence des bactéries. Le but de notre travail était donc d'évaluer cette modulation de virulence dans les différents stress rencontrés par *S. aureus* au sein d'une plaie du pied chez un patient diabétique. Nous avons testé 3 conditions particulières : l'hyperglycémie caractéristique du diabète déséquilibré, l'ischémie qui est une des complications majeures du diabète et systématiquement présente dans les plaies du pied chez le diabétique et la présence d'antibiotiques à des concentrations sub-inhibitrices tels que la vancomycine qui inhibe la synthèse des peptidoglycanes de la paroi et le linézolide dont le rôle est d'inhiber la synthèse protéique.

L'étude de l'adaptation du *S. aureus* aux conditions environnementales de stress rencontrées dans le pied diabétique a été effectuée dans un modèle *in vitro* que nous avons adapté pour mimer ces conditions de chronicité rencontrées dans le pied diabétique. Ce modèle a pu être

« validé » par l'apparition dans toutes les conditions environnementales testées de small colony variants (SCVs) qui sont de petites colonies métaboliquement inactives qui n'induisent pas de réponse immunitaire de l'hôte et qui sont caractéristiques de *S. aureus* en situation d'infection persistante ^{117,118}.

Les études transcriptomiques par qRT-PCR après 24h de culture correspondant à une situation d'infection aiguë, ont montré une surexpression des gènes de virulence. Par exemple les gènes codant pour la production de la toxine de Panton et Valentine étaient surexprimés dans notre modèle quelle que soit les conditions testées. Toutes les études n'ont pas obligatoirement montré cet effet comme celle d'Otto *et al.* ⁴⁷ qui observait plutôt une diminution de la production de PVL sous l'effet du linézolide. Néanmoins, l'étude de Dumitrescu ⁴⁵ a montré que le linézolide pouvait induire différents effets dépendant de la concentration et de la souche de *S. aureus*.

Après une exposition prolongée (16 semaines) à ces conditions de stress, une forte diminution de l'expression des gènes codant pour la PVL et EdinB a été notée dans toutes les conditions testées : diminution de « fold change » de *lukF/lukS-PV* de 3 fois sous vancomycine, 7 fois dans un milieu hyperglucosé, 8 fois en anaérobiose, et 2 fois sous linézolide. Les « fold changes » d'*edinB* étaient diminués de 10 fois en anaérobiose, 14 et 20 fois sous vancomycine et linézolide, respectivement. Pour *edinC*, des diminutions de 15 fois en anaérobiose et sous linézolide, et de 20 et 32 fois sous vancomycine et en milieu hyperglucosé ont été observées, respectivement. Concernant les autres toxines, l'expression du gène codant pour l'hémolysine beta (*hly*) était diminuée dans toutes les souches quelle que soit la condition testée. L'expression du gène codant pour l'Hla était diminuée en anaérobiose pour les souches NSA739 et 7475, mais surexprimé en milieu hyperglucosé pour ces deux souches. Le gène codant pour l'entérotoxine Sea était également sous exprimée sous vancomycine pour la souche 1077, sous linézolide et en anaérobiose pour la souche NSA739. Cependant le gène *sea* était surexprimé sous vancomycine pour NSA739 et 7475 et sous linézolide pour 1077 et 7475.

L'ensemble de ces résultats suggère que l'expression des principaux gènes de virulence du *S. aureus* est sous-exprimée après une exposition prolongée à des concentrations sub-inhibitrices d'antibiotiques, en anérobiose et en hyperglycémie. Ces résultats ont été notamment observé sur la souche 7475. Il est intéressant de noter que l'addition d'antibiotiques après 16 semaines de culture en excès de glucose a induit la surexpression des gènes *fnbA*, *eap*, *hla* dans la souche NSA739. Toutefois, dans notre modèle *in vivo* de nématodes, ce résultat n'était pas confirmé puisqu'une réduction de virulence était observée. L'expression de ces gènes ne

suffisait pas à augmenter la virulence de la souche. Nous avons également observé que l'addition de vancomycine dans une souche cultivée pendant de longues semaines dans ces conditions de stress, ne changeait pas l'expression des gènes qui étaient déjà affectée (comme par exemple pour la souche 1077). Par contre, l'addition de linézolide avait un effet notable sur *edinB* qui était surexprimé contrairement aux autres gènes qui étaient toujours sous-exprimés. Les études *in vivo* sur *C. elegans* ont montré une diminution de la virulence de cette souche (1077) après addition du linézolide, confirmant les résultats et montrant que *edinB* seul surexprimé ne suffisait à entraîner une augmentation de la virulence bactérienne. Nos résultats semblent donc indiquer que les antibiotiques (vancomycine et linézolide) diminuent de façon significative la virulence des souches de *S. aureus*. Le linézolide s'est avéré être plus efficace que la vancomycine. Ce résultat est tout à fait conforme à ce qui avait déjà été montré par d'autres études¹¹⁹⁻¹²² dont les conclusions étaient que le linézolide était d'une meilleure efficacité clinique que la vancomycine. Les tests pour mesurer la formation de biofilm ont montré une réduction de cette formation dans toutes les souches après une longue exposition au glucose et à la vancomycine. Cependant, l'addition de vancomycine après 16 semaines dans un milieu hyperglucosé induit un regain de formation de biofilm.

Tous ces résultats nous confirment que la virulence du *S. aureus* est très impactée par les différentes conditions rencontrées au niveau du pied diabétique, en particulier les gènes codant pour les toxines PVL et Edin. Cependant, les souches de *S. aureus* essaient de survivre soit par l'activation d'autres facteurs de virulence (comme les toxines Sea, Hla), soit par un changement phénotypique en small colony variant, soit enfin par la formation de biofilm.

Ces résultats très intéressants permettent pour la première fois de mettre en évidence l'influence de certains facteurs environnants dans le pied diabétique sur les souches infectantes de *S. aureus*. La qRT-PCR ne nous permet pas cependant de savoir quelle est la quantité réelle de toxine produite par ces bactéries. Des tests Elisa pourraient ainsi être entrepris. De plus, cette étude a été réalisée sur un modèle *in vitro* utilisant les concentrations subinhibitrices d'antibiotiques. La pharmacocinétique de ces antibiotiques dans le pied n'étant pas connue, il serait intéressant d'effectuer des études dans ce sens pour connaître les concentrations réelles d'antibiotiques arrivant au niveau du pied diabétique et ainsi travailler dans les conditions réelles. Dans ce sens, il serait donc intéressant d'effectuer cette étude sur un modèle *in vivo* tel que les souris diabétiques. Un panel plus important de souches cliniques pourraient également être testé. Ceci rapprocherait plus des conditions humaines. D'autres part, nous avons montré qu'après plusieurs semaines de culture certains gènes d'adhésion pouvaient être impactés. Il

conviendrait donc d'étudier la capacité d'adhésion de ces souches sur un modèle *ex vivo* de cellules humaines.

En dehors des conditions stringentes du pied diabétique, la virulence de *S. aureus* peut être influencée par les interactions bactériennes qui sont également un élément majeur à prendre en compte. En effet, les infections des plaies aiguës ou chroniques sont souvent polymicrobiennes⁹⁷. Il a été montré récemment qu' *H. kunzii* qui est une bactérie commensale, était très fréquemment associée à *S. aureus* dans les plaies du pied chez le diabétique. Ainsi, nous avons décidé d'étudier les effets de l'association entre *H. kunzii* et *S. aureus*.

Le modèle *in vivo* utilisé est le nematode *C. elegans*. Il s'agit d'un modèle permettant d'étudier la virulence des pathogènes. Depuis maintenant plusieurs années de très nombreuses publications utilisent ce modèle pour cribler la virulence bactérienne. De plus, même s'il n'est pas extrêmement développé, cet organisme met en place des mécanismes de défense immunitaires pour combattre les infections^{123–125}. Pour cela, il utilise des voies métaboliques ayant des similitudes et ou des homologues avec ceux des vertébrés et des humains pendant l'infection. Par exemple il utilise le facteur de transcription clé *hlh-30*, homologue du gène TFEB chez le mammifère connu pour son importance pour l'autophagie et la biosynthèse lysosomale^{123,126}. Par ailleurs, il a été montré que les bactéries utilisaient les mêmes mécanismes de virulence sur *C. elegans* que lors de l'infection sur les mammifères. Pour ce qui est de *S. aureus* par exemple, Sifri *et al.* ont montré l'importance des régulateurs *agr*, *sar*, *sigB*, de la protéase V8 et de l'alpha toxine pour induire la mort des nématodes¹²⁷.

Ce modèle a été utilisé dans notre étude pour évaluer la virulence de souches d'*H. kunzii*. Grâce à ce modèle, nous avons montré que 74% des souches d'*H. kunzii* testées étaient non virulentes, et 26% étaient très peu virulentes ; tandis que les souches de *S. aureus* étaient virulentes. Ce résultat est en parfait accord avec ce qui a déjà précédemment été montré c'est à dire que les souches d'*H. kunzii* sont des souches non virulentes¹²⁸ mais certaines d'entre elles peuvent être des pathogènes opportunistes^{109,129–131}. Plus encore, l'analyse de l'expression des gènes de défense de l'hôte a montré qu'*H. kunzii* n'induisait pas l'expression des gènes de défense de l'hôte contrairement à *S. aureus* qui entraînait la production du facteur de transcription global *hlh-30*, du gène antimicrobien *lys-5* et du gène de détoxification *sodh-1*. Ces résultats sont en accord avec ceux de deux précédentes études qui ont montré une production

des gènes de défense de *C. elegans* (*clec-71*, *sodh-11*, *cyp-37B1*, *lys-5*) 8h après une infection avec le *S. aureus* ^{123,124}.

Certaines interactions entre *S. aureus* et différentes bactéries ont été précédemment décrites comme la coopération avec *E. faecalis* et *Candida albicans*,^{98,99}, la compétition avec *Lactobacillus* sp.¹⁰⁰ et *Streptococcus pneumoniae* ¹⁰¹, mais aucune n'a été faite entre le *S. aureus* et *H. kunzii*. Dans cette étude, nous avons observé que toutes les souches d'*H. kunzii* inhibaient la virulence des souches de *S. aureus* (LT50s: 4.1 to 5.7 vs 2.8 days, $P < 0.001$) ouvrant donc la réflexion sur deux hypothèses: *H. kunzii* module la réponse immunitaire de l'hôte le rendant plus résistant ou tolérant à *S. aureus* ou alors *H. kunzii* agit directement sur la virulence de *S. aureus* ?

De précédentes études avaient déjà démontré qu'une primo infection avec *S. aureus* augmentait la vulnérabilité de *C. elegans* et modifiait sa tolérance au pathogène opportuniste *Proteus mirabilis* ¹³². Cependant, les infections séquentielles réalisées avec les souches d'*H. kunzii* nous ont montré que cette bactérie n'affectait pas la tolérance de *C. elegans* à *S. aureus*. Ainsi, nous avons émis l'hypothèse qu'*H. kunzii* agissait probablement directement sur la virulence des souches de *S. aureus*. Les expériences transcriptomiques menées sur les gènes de virulence de *S. aureus* nous ont notamment montré une diminution d'expression du gène régulateur global de la virulence de *S. aureus* pouvant expliquer la baisse de la virulence de cette bactérie.

Ces résultats nous ont permis de montrer pour la première fois une diminution de la virulence d'une souche naturellement pathogène par une souche commensale non pathogène. Cette étude est d'un grand intérêt mais plus d'expériences devraient être menées sur des modèles plus complexes d'études tels que les souris pour se rapprocher encore plus de l'humain. De plus des études transcriptomiques plus complètes telles que le RNAseq, les microarrays devraient être réalisées sur *S. aureus* en présence d'*H. kunzii* pour essayer d'avoir une vision globale du génome afin de voir quels sont les gènes et les voies métaboliques réellement impactées par *H. kunzii*. Plus encore, des études du génome d'*H. kunzii* pourraient être réalisées pour identifier les facteurs responsables de l'induction de cette baisse de virulence. Il serait aussi intéressant de voir si *H. kunzii* pourrait également affecter la virulence d'autres espèces pathogènes.

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ANNEXES

Résumé :

Le diabète sucré est un problème majeur de santé publique. L'une de ses principales complications est l'ulcère du pied (UPD) à l'origine d'infections et de nombreuses amputations. Ces plaies sont majoritairement polymicrobiennes. La principale bactérie isolée est *Staphylococcus aureus* (SA) qui pose d'importants problèmes en thérapie courante. Bactérie commensale notamment des fosses nasales, elle est un vrai pathogène au niveau des UPD. La compréhension du passage du commensalisme à la pathogénicité reste peu connue. Or récemment une souche colonisante de SA présentant un faible potentiel de virulence, et isolée sur un UPD a été identifiée. Cette souche présente un phage (ROSA-like) inséré dans son génome dont la stabilité a été démontrée. Ce phage est responsable de la faible virulence de cet isolat.

Ce travail de doctorat a permis : i) de mettre en évidence l'implication du phage ROSA-like dans la dérégulation des mécanismes d'acquisition du fer, mécanismes essentiels pour la survie du SA ainsi que dans le métabolisme global de cette souche. Ces résultats ont confirmé le rôle clé du phage dans la baisse de la virulence du SA colonisant ; ii) à partir d'un modèle in vitro d'infections chroniques mimant les conditions rencontrées dans les UPD, d'étudier le comportement de souches cliniques de SA (colonisantes/infectantes) en fonction des différentes conditions environnementales : hyperglycémie, anaérobiose, antibiotiques (linézolide et vancomycine). Les résultats ont montré qu'une exposition prolongée (plus de 24 semaines) à ces conditions de stress notamment aux antibiotiques, réduisait la virulence des souches de SA ; iii) d'évaluer la virulence des SA en présence d'autres bactéries qui l'entourent, élément essentiel du polymicrobisme de l'UPD. Ce travail s'est particulièrement intéressé aux interactions existant entre SA et *Helcococcus kunzii*, bactérie fréquemment isolée en association avec le SA dans les UPD. Il a montré pour la première fois une baisse de la pathogénicité des souches de SA par une bactérie commensale non virulente.

Mots clés : *Staphylococcus aureus*, pied diabétique, virulence, infection, colonisation, phage.

Abstract:

Diabetes mellitus is a global problem. One of the main complications is diabetic foot ulcer (DFU) which can evolve towards infection and induces lower limb amputations. Those wounds are mostly polymicrobial and *Staphylococcus aureus* (SA), the most prevalent pathogen isolated in this situation causes numerous therapeutic problems. This bacterium, known to be a commensal organism present notably in nasal cavity, is a real pathogen in DFU. However, the comprehension between the commensalism and the pathogenicity of SA remains still unknown. Recently, a colonizing SA strain with very low virulence potential was discovered in DFU. This strain possesses a phage named Rosa-like responsible of its low virulence.

This work helped us: i) to highlight the involvement of the Rosa-like phage in the dysregulation of iron uptake mechanisms, the main source for SA survival and its global metabolism. The obtained results confirm the key role of the phage in the decrease of the colonizing SA strain virulence; ii) to study the behaviour of clinical SA strains (colonizing/infecting) using an in vitro chronic infection model miming different environmental conditions encountered in DFU: hyperglycemia, anaerobic condition, antibiotics (linezolid and vancomycin). Results showed that prolonged exposure (up to 24 weeks) to those stress conditions notably antibiotics reduced SA strains virulence; iii) to evaluate SA virulence in presence of other bacteria present in DFU. Particularly, in this work a focus was performed in the interaction between SA and *Helcococcus kunzii*, gram positive bacteria frequently isolated with SA in DFU. It was shown for the first time a decrease of SA pathogenicity by a commensal non virulent bacterium.

Key words: *Staphylococcus aureus*, diabetic foot, virulence, infection, colonization, phage.