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# sRNA-controlled iron-sparing response in Staphylococci

Rodrigo Hernán Coronel Téllez

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sRNA-controlled iron-sparing response in  
*Staphylococci*

Adaptation à la carence en fer chez les Staphylocoques  
par l'ARN régulateur IsrR

**Thèse de doctorat de l'université Paris-Saclay**

École doctorale n° 577, Structure et Dynamique des Systèmes Vivants (SDSV)  
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Référent : Faculté des Sciences d'Orsay

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## B. RÉSUMÉ DE LA THÈSE

 université  
PARIS-SACLAY

ÉCOLE DOCTORALE

Structure et dynamique  
des systèmes vivants  
(SDSV)

**Titre :** Adaptation à la carence en fer chez les Staphylocoques par l'ARN régulateur IsrR

**Mots clés :** *Staphylococcus aureus*, ARN non-codant, Métabolisme de nitrates, RyhB, Fer, Virulence

**Résumé :** Le fer est un élément essentiel pour la plupart des organismes vivants en raison de son rôle important dans de nombreux processus enzymatiques ; cependant, sa biodisponibilité est relativement faible. Chez les bactéries, il a été démontré que les sRNAs (*small RNAs*, petits ARNs) sont des régulateurs importants de l'homéostasie du fer, l'exemple le plus connu étant celui de RyhB chez *E. coli*. RyhB est le principal régulateur de la réponse d'épargne de fer, qui consiste en la régulation négative des ARNm codant pour des protéines non essentielles utilisant de fer lorsque cet élément est rare, de cette façon, le peu de fer disponible peut être redirigé vers des processus plus vitaux. Jusqu'à présent, aucun sRNA dictant ce processus n'a été décrit dans l'important pathogène humain *Staphylococcus aureus*.

Par conséquent, l'objectif de cette thèse est d'élucider la réponse d'épargne de fer régulée par des sRNAs chez *S. aureus*, un mécanisme d'adaptation d'importance vitale pour faire face à la privation de fer chez l'hôte.

Un seul sRNA, nommé ici IsrR, a émergé d'un test de compétition avec des bibliothèques de sRNAs mutants comme étant essentiel pendant la carence de fer. IsrR est réprimé en fonction du fer par le régulateur Fur, et prédit qu'il cible les ARNm exprimant des enzymes contenant du fer. Parmi celles-ci, IsrR régule négativement les ARNm des enzymes qui catalysent la respiration anaérobie des nitrates. IsrR est nécessaire pour la létalité de *S. aureus* dans un modèle murin de septicémie, soulignant son rôle en tant que contributeur majeur à la réponse d'épargne de fer pour la survie bactérienne pendant l'infection. IsrR est conservé parmi les staphylocoques, mais il est structurellement et mécaniquement distinct du sRNA RyhB. Il est remarquable que ces sRNA distincts régulent des cibles communes, ce qui permet de conclure que la régulation basée sur les sRNA fournit des solutions évolutives optimales pour améliorer la capacité bactérienne lorsque le fer est rare.

## C. SUMMARY OF THE THESIS

université  
PARIS-SACLAY

ÉCOLE DOCTORALE

Structure et dynamique  
des systèmes vivants  
(SDSV)

**Title :** sRNA-controlled iron-sparing response in Staphylococci

**Keywords :** *Staphylococcus aureus*, Non-coding RNA, Nitrate metabolism, RyhB-like, Iron, Virulence

**Abstract :** Iron is an essential element for most living organisms due to its important role in numerous enzymatic processes; however, its bioavailability is relatively low. In bacteria, sRNAs (small RNAs) are important regulators of iron homeostasis with RyhB in *E. coli* as the best-known example. RyhB is the main regulator of the so-called iron-sparing response, which consist in the downregulation of mRNAs encoding non-essential proteins using iron when this element is scarce, in this way, the few available iron can be re-directed to more vital processes. So far, no sRNA dictating his process has been described in the important human pathogen *Staphylococcus aureus*.

Therefore, the objective of this thesis is to elucidate the sRNA-regulated iron-sparing response in *S. aureus*, an adaptation mechanism of vital importance to cope with iron deprivation within the host.

A single sRNA, named here IsrR, emerged from a competition assay with sRNA-mutant libraries as being essential in iron starvation. IsrR is iron-repressed through the ferric uptake regulator (Fur) and predicted to target mRNAs expressing iron-containing enzymes. Among them, IsrR down-regulates mRNAs of enzymes that catalyze anaerobic nitrate respiration. IsrR is required for the lethality of *S. aureus* in a mouse model of sepsis, underscoring its role as a major contributor to the iron-sparing response for bacterial survival during infection. IsrR is conserved among staphylococci, but structurally and mechanistically distinct from the sRNA RyhB. Remarkably, these distinct sRNAs regulate common targets, leading to the conclusion that RNA-based regulation provides optimal evolutionary solutions to improve bacterial fitness when iron is scarce.

## D. LONG RÉSUMÉ DE LA THÈSE

### 1. Introduction

Le fer est l'un des métaux les plus abondants sur la Terre et c'est un élément essentiel pour la plupart des êtres vivants, y compris les bactéries. En raison de son potentiel redox élevé, il est impliqué dans de nombreux processus biologiques vitaux, tels que la biosynthèse de l'ADN, le cycle du citrate, ou la respiration anaérobie, où il se trouve principalement comme cofacteur de nombreuses enzymes, ou comme composant de centres fer-soufre (Fe-S) ou de groupes hème.

Le fer existe sous deux formes, le fer ferreux ( $\text{Fe}^{2+}$ ) ou ferrique ( $\text{Fe}^{3+}$ ).  $\text{Fe}^{2+}$  est la principale forme de fer utilisée par les bactéries ; cependant, il est peu disponible dans la plupart des environnements. Pour les bactéries pathogènes, l'acquisition du fer est une étape cruciale lors de l'infection pour assurer leur survie et dissémination à l'intérieur de l'hôte.

Pour éviter la prolifération bactérienne, l'hôte humain limite le fer libre. Par exemple, en incorporant cet élément dans l'hème au sein des érythrocytes, ou dans des protéines qui stockent ou transportent le fer comme la ferritine ou la transferrine. Ce processus est connu sous le nom d'immunité nutritionnelle. En réponse, les bactéries pathogènes ont développé diverses stratégies pour surmonter cette limitation et « voler » le fer de l'hôte. Par exemple, les hémolysines bactériennes endommageront les globules rouges et rendront le fer lié à l'hème plus accessible. Les sidérophores, molécules à très forte affinité pour le fer, peuvent extraire le fer de la ferritine et être captés par les bactéries. Enfin, les récepteurs de la transferrine dans la membrane cellulaire bactérienne peuvent extraire le fer directement de cette protéine de l'hôte. Il y a en permanence une « bataille du fer » entre les bactéries pathogènes et l'hôte.

Les bactéries doivent assurer une homéostasie adéquate du fer dans la cellule. Alors que la carence en fer affecte la croissance bactérienne, son excès peut être toxique. Par conséquent, l'équilibre entre l'acquisition du fer et son utilisation est finement régulé. De nombreux processus cellulaires impliqués dans l'homéostasie du fer sont contrôlés par le régulateur d'absorption ferrique Fur (*ferric uptake regulator*), qui, dans des conditions riches en fer, inhibera la transcription des gènes principalement impliqués dans l'acquisition du fer. Fur se lie à des motifs spécifiques de régions promotrices, bloquant l'entrée de l'ARN polymérase.

Cependant, dans des conditions de carence en fer, Fur est inactif permettant la transcription des gènes qu'il réprime.

Les ARN régulateurs sont des régulateurs importants de nombreux processus bactériens, y compris de l'homéostasie du fer. Généralement, les ARN régulateurs bactériens (ARNrég) ont une taille comprise entre 50 et 250 nucléotides (nt) et ne codent pas pour une protéine. Pour cette raison, ils sont également connus sous le nom de « petits ARN ». Cependant, il existe quelques exceptions telles que RNAIII chez *Staphylococcus aureus*, qui a une taille inhabituellement longue de 514 nt et code pour l'hémolysine delta.

Les ARNrég peuvent se lier à des protéines ou à d'autres ARN, le plus souvent des ARN messagers (ARNm) pour réguler leur activité. Selon la région génomique où les ARNrég sont codés par rapport à leur cible, ils peuvent être classés comme codés en *cis* ou en *trans*. Les ARNrég codés en *trans* ont leurs loci dans un site distant de leur ARNm cible avec lequel ils partagent une complémentarité nucléotidique limitée. Les ARNrég ont de nombreux mécanismes de régulation différents sur leurs ARNm cibles. Les plus courants sont les suivants : a) inhiber la traduction de l'ARNm après l'appariement des bases au site de liaison du ribosome ; b) induire la dégradation de l'ARNm (et lui-même) après le recrutement des RNases ; c) faciliter la traduction par appariement des bases aux régions bloquant le site de liaison au ribosome, et d) favoriser la stabilité d'ARN cible par la formation de complexes non reconnus par certaines RNases.

Étant donné que la plupart des ARNrég sont exprimés dans des conditions de croissance spécifiques et qu'ils agissent principalement au niveau post-transcriptionnel, les ARNrég exercent souvent une régulation fine, avec des phénotypes généralement discrets. Les ARNrég se lient généralement à plusieurs ARNm cibles et régulent de nombreux mécanismes cellulaires, tels que la virulence, la résistance aux antibiotiques et l'homéostasie du fer.

L'ARNrég le mieux étudié impliqué dans l'homéostasie bactérienne du fer est RyhB, un ARNrég de 90 nt découvert pour la première fois chez *E. coli*. La transcription de *ryhB* est réprimée par Fur, il n'est donc exprimé que dans des conditions de carence en fer. Cet ARNrég a besoin de la protéine chaperon d'ARN Hfq pour se lier et réguler ses ARNm cibles.

La fonction principale de RyhB est l'inhibition des ARNm codant pour des protéines non essentielles qui utilisent ou contiennent du fer, lorsque cet élément est rare. Ainsi, le peu de fer

disponible est redirigé vers des processus cellulaires plus vitaux. Cette stratégie d'adaptation est connue sous le nom de réponse d'épargne du fer.

Les orthologues de RyhB, qui sont des gènes avec une séquence nucléotidique très similaire à RyhB, sont présents chez de nombreuses espèces de la famille des entérobactéries où ils contribuent à l'adaptation aux conditions de carence en fer. Chez les espèces de *Pseudomonas*, un acteur majeur de cette adaptation est PrrF, un ARNrég inhibé par Fur qui contribue à l'homéostasie du fer. Étonnamment, on en sait beaucoup moins sur les bactéries Gram-positives où, jusqu'à présent, les orthologues de RyhB et PrrF n'ont pas été trouvés. Les ARNrég peuvent néanmoins être des régulateurs de la réponse d'épargne du fer. Chez *Bacillus subtilis*, l'ARNrég FsrA est inhibé par Fur, et avec l'aide de trois chaperons d'ARN, FbpA, FbpB et FbpC, il empêche l'expression d'enzymes contenant du fer. Chez *Mycobacterium tuberculosis*, l'ARNrég MrsI est apparemment inhibé par IdeR, un homologue fonctionnel de Fur, et exerce une réponse similaire d'économie du fer. De façon intéressante, cette bactérie ne possède pas Hfq. Une question émergente de ces dernières années, est l'identification d'ARNrég remplissant ce mécanisme de régulation chez d'autres bactéries, en particulier chez des agents pathogènes humains comme *Staphylococcus aureus*.

*S. aureus* est une bactérie Gram-positif avec un génome riche en AT. C'est un anaérobie facultatif qui en l'absence d'oxygène peut utiliser le nitrate comme accepteur final d'électrons ou basculer son métabolisme vers la fermentation. C'est un agent pathogène humain important qui peut provoquer un large éventail de maladies, des infections cutanées non graves à des affections potentiellement mortelles telles que la bactériémie ou l'endocardite. Son succès en tant qu'agent pathogène est dû en partie à la production de nombreux facteurs de virulence et à une adaptation impressionnante à divers environnements. Ces dernières années, il a été découvert que ces processus sont souvent régulés par des ARNrég tels que RNAIII ou RsaE. Cependant, aucun ARNrég impliqué dans l'homéostasie du fer n'avait été décrit.

## 2. Objectif

Identifier et caractériser les ARNrég contribuant à la régulation de l'homéostasie du fer chez *S. aureus*, un processus d'une importance vitale lors de l'infection.

### 3. Résultats

**IsrR est un ARNrég nécessaire à la croissance dans les milieux appauvris en fer.** Pour savoir si les ARNrég sont nécessaires chez *S. aureus* pour s'adapter à une faible concentration en fer, j'ai profité d'une stratégie développée dans notre laboratoire, basée sur l'utilisation d'une bibliothèque de mutants d'ARNrég. Des mutants de délétion marqués à l'ADN construits dans la souche HG003 ont été assemblés dans des bibliothèques indépendantes. La collection comprend 48 mutants ayant chacun une délétion d'un gène exprimant un « *bona fide* ARNrég » (définis comme ceux qui sont génétiquement indépendants avec leur propre promoteur et terminateur) ; on s'attend à ce que la délétion et la substitution par des séquences d'étiquettes de ces gènes n'affectent pas l'expression des gènes adjacents. La région supprimée de chaque mutant a été remplacée par une étiquette ADN spécifique (*tag*), qui permet de compter chaque mutant et donc d'évaluer leur proportion au sein de la population. Les mutants qui disparaissent ou s'accumulent dans une condition de stress donnée donne une indication sur le rôle fonctionnel des ARNrég correspondants. Les bibliothèques ont été confrontées à un milieu carencé en fer par l'ajout de chélateurs de fer, le 2,2'-dipyridyle (DIP) et l'acide éthylènediamine-N,N'-bis(2-hydroxyphényl)acétique (EDDHA). La proportion de chaque mutant au sein de la population a été déterminée à différentes étapes de croissance. Les résultats ont été normalisés au même milieu sans chélateur de fer. Parmi les 48 mutants testés, la souche avec le tag135 présentait un désavantage significatif en termes de fitness avec les deux chélateurs de fer testés ; après environ 28 générations, sa distribution par rapport à la condition de contrôle a diminué de plus de 10 en DIP et de 1000 fois en EDDHA.

La délétion associée au tag135 inactive un ARNrég rapporté dans deux études sous les noms S596 et Tsr25. Il a été renommé ici IsrR pour *iron-sparing response regulator* (régulateur de la réponse d'épargne du fer). La souche HG003  $\Delta$ *isrR* n'avait aucun défaut de croissance apparent en milieu riche par rapport à sa souche parentale. Cependant, le mutant *isrR* présentait un retard de croissance en présence de DIP et d'EDDHA, confirmant les résultats de l'expérience de fitness et démontrant que le gène  $\Delta$ *isrR* est nécessaire pour une croissance optimale dans un milieu carencé en fer. Il est à noter que le léger retard de croissance de  $\Delta$ *isrR* observé correspond à un coût de fitness drastique quand ce mutant est en compétition avec d'autres bactéries. Pour confirmer que le phénotype était uniquement dû à l'absence d'IsrR, une souche  $\Delta$ *isrR* a été complétée par une copie fonctionnelle d'*isrR* avec son promoteur endogène. La souche  $\Delta$ *isrR* résultante portant une seule copie du gène *isrR* avaient des taux de croissance équivalents



à celui de la souche parentale lorsqu'elles étaient cultivées avec DIP ou EDDHA. Ces résultats confirment que le phénotype  $\Delta isrR$  observé était strictement dépendant de *isrR*.

**L'expression d'IsrR est réprimée par Fur.** Les extrémités d'IsrR ont été déterminées par des expériences 5'/3'RACE. La transcription d'*isrR* génère un ARN<sub>rég</sub> avec une longueur de 174 nucléotides. La présence d'une boîte de liaison à Fur dans la région du promoteur, suggèrent que l'expression d'*isrR* serait dépendante du fer. IsrR n'est pas été détecté par Northern blot en milieu riche dans la souche parentale. En revanche, *isrR* est fortement exprimé dans le mutant  $\Delta fur$ , démontrant que Fur contrôle négativement l'expression d'*isrR*. Le gène *isrR* est apparemment contrôlé par deux boîtes Fur, une dans la région du promoteur et la seconde immédiatement après le site de démarrage de la transcription. Pour tester leurs contributions relatives, un système de rapporteur transcriptionnel sous le contrôle du promoteur *isrR*, a été construit. La contribution de chaque boîte Fur prédite a été testée par mutagenèse en modifiant le premier, le deuxième ou les deux motifs de liaison à Fur et il a été observé que les deux sites contribuent à une répression efficace d'*isrR* dépendante de Fur, le premier étant épistatique sur le deuxième.

**IsrR et sa régulation par Fur sont conservés dans le genre *Staphylococcus*.** La plupart des ARN<sub>rég</sub> bactériens agissant en *trans* sont peu conservés d'une espèce à l'autre, y compris parmi les staphylocoques. Cependant, le gène *isrR* a été détecté *in silico* dans tous les génomes analysés du genre *Staphylococcus* et la conservation des séquences comprend les deux boîtes Fur.

Les orthologues d'IsrR de différentes espèces du genre *Staphylococcus* ont été utilisés pour alimenter LocARNA, un logiciel d'alignements multiples d'ARN qui génère des prédictions de structure secondaire et met en évidence les régions d'appariement conservées. La structure d'IsrR proposée comprend trois tiges-boucles et un terminateur canonique indépendant de Rho. Trois régions riches en C (CRR, *C-rich regions*), nommées ici CRR1 à 3, ont été prédites monocaténares et conservées. Les motifs riches en C sont importants pour la reconnaissance directe des ARN<sub>rég</sub>/cibles chez *S. aureus*. Cette caractéristique discrimine efficacement les cibles dans les souches de staphylocoques car leurs génomes sont riches en AT (environ 70%), et les segments riches en G sont rares sauf dans les séquences Shine-Dalgarno. Deux des trois tiges ont conservé des séquences primaires avec peu de variation, indiquant l'importance de chaque nucléotide, peut-être pour une interaction avec une protéine jusqu'à présent inconnue.

L'expression et la dépendance au fer d'IsrR ont été confirmées expérimentalement pour *S. haemolyticus*, *S. epidermidis* et *S. lugdunensis*. Les résultats suggèrent qu'IsrR a une fonction importante liée au métabolisme du fer qui est conservée dans tout le genre *Staphylococcus*. La présence de CRR suggère qu'IsrR est probablement un ARNrég agissant en *trans*.

**Les prédictions bioinformatiques indiquent qu'IsrR cible des ARNm codant pour des enzymes avec des centres Fe-S.** Il est possible de prédire les cibles putatives des ARNrég bactériens agissant en *trans* par bioinformatique. CopraRNA est un prédicteur efficace de cibles des ARNrég lorsque des séquences orthologues d'un ARNrég sont détectées parmi différentes espèces comme c'est le cas pour IsrR au sein du genre *Staphylococcus*. Les résultats indiquent une convergence fonctionnelle des cibles proposées. Parmi les 23 meilleurs candidats, 7 sont des ARNm qui expriment des protéines contenant un centre Fe-S (NasD, MiaB, GltB2, FdhA, CitB, NarG et SAOUHSC\_01062). Considérant 32 protéines différentes contenant un centre Fe-S dans *S. aureus* lors d'une analyse d'expert, la probabilité d'identifier par hasard 7 ARNm codant pour une protéine contenant un centre Fe-S est de  $2 \times 10^{-9}$ . Cette probabilité remarquablement faible permet d'affirmer que CopraRNA a identifié des cibles directes pertinentes. D'autres cibles putatives, telles que les ARNm *moaD* et *nreC*, sont également associées à des complexes contenant du Fe-S. Il est donc probable que la plupart, sinon la totalité, des cibles prédites associées aux centres Fe-S soient des cibles directes d'IsrR. La nitrate réductase, la nitrite réductase, la glutamate synthase, la formate déshydrogénase, l'aconitate et l'hydratase méthionine sulfoxyde réductase qui sont apparemment affectées par IsrR, correspondent également à des enzymes putatives ou démontrées affectées par RyhB suggérant une convergence fonctionnelle frappante entre ces deux ARNrég.

**IsrR cible des ARNm codant pour les enzymes liées au métabolisme de nitrates.** En l'absence d'oxygène, *S. aureus* utilise le nitrate, s'il est disponible, comme accepteur d'électrons de la chaîne respiratoire. Le nitrate est converti en nitrite par la nitrate réductase codée par l'opéron *nar*. Dans une deuxième étape, le nitrite est converti en ammonium par la nitrite réductase codée par l'opéron *nas*. L'ammonium est ensuite utilisé par la glutamate synthase codée par le gène *gltB2*. La transcription des opérons *nar* et *nas* dépend du système de régulation NreABC, dont son activité nécessite la présence de nitrate et l'absence d'oxygène. Étonnamment, les ARNm *narG* (sous-unité de la nitrate réductase), *nasD* (grande sous-unité de la nitrite réductase) et *gltB2* sont des cibles putatives d'IsrR, suggérant qu'IsrR pourrait affecter toutes les étapes de la conversion du nitrate en glutamate. Il a été suggéré que chez *E. coli*, la formate déshydrogénase, codé par l'ARNm *fdhF*, est associée à la voie de métabolisme des nitrates, et il

est intéressant de noter que l'orthologue *fdhF* de *S. aureus*, *fdhA*, devrait également être une cible d'IsrR.

Les ARNm *fdhA*, *narG*, *nasD* et *gltB2* associées à la voie de métabolisme des nitrates ont été retenus comme cibles putatives d'IsrR. Une analyse bioinformatique suggère qu'IsrR s'associe par appariement de bases au niveau des séquences Shine-Dalgarno de ces ARNm et que les CRR d'IsrR sont impliqués dans cet appariement.

Dans de nombreux cas, l'interaction d'un ARNrég avec son ARNm cible entraîne la déstabilisation des transcrits. Pour cela, la stabilité des ARNm *fdhA* et *gltB2* a été évaluée lors de l'inhibition de la transcription par la rifampicine. Dans HG003 et son dérivé  $\Delta$ *isrR*, les ARNm *fdhA* et *gltB2* sont instables en milieu riche. L'ajout de DIP au milieu qui induit l'expression de *isrR* n'a entraîné des changements de la stabilité des ARNm *fdhA* et *gltB2*.

Pour mettre en évidence une liaison d'IsrR avec des cibles putatives liées aux nitrates, la formation d'un complexe entre IsrR et le ARNm *fdhA* a été testé par retard sur gel (EMSA, *electrophoretic mobility shift assay*). Un décalage de la bande correspondant à IsrR a été observé en présence d'ARNm *fdhA* suggérant la formation d'un complexe IsrR/ARNm *fdhA*.

Pour étudier plus en détail la liaison directe entre IsrR et les cibles ARNm liées aux nitrates *fdhA*, *narG*, *nasD* et *gltB2*, une méthode expérimentale appelée SHAPE sondant la réactivité des nucléotides individuels à l'anhydride 1-méthyl-7-nitroisatoïque (1M7) associée à l'accessibilité des nucléotides a été réalisée. La réactivité d'IsrR et de chaque cible putative sélectionnée a été évaluée lorsqu'ils étaient seuls ou incubés en tant que paire ARNrég/ARNm. Des variations de réactivité sur des nucléotides spécifiques ont été observées pour toutes les cibles d'IsrR en les comparant en présence ou en l'absence d'IsrR. Ces données soutiennent une interaction d'IsrR avec les quatre cibles ARNm putatives testées. En outre, une structure secondaire d'IsrR putative combinant les données de stabilité thermodynamique des paires de bases intramoléculaires et les résultats de la réactivité a été proposée.

**Contrôle traductionnel par IsrR.** La présence de CRR, les prédictions d'appariement et les résultats de SHAPE suggèrent qu'IsrR affecte la traduction des cibles ARNm sélectionnées. Pour étudier les interactions IsrR-ARNm et déterminer l'implication des CRR d'IsrR dans la régulation des cibles, j'ai utilisé un système rapporteur traductionnel. Cette stratégie m'a permis aussi de valider les interactions des CRR d'IsrR avec les ARNm *fdhA*, *narG*, *nasD* et *gltB2*. À cette fin, les

séquences 5'UTR de chaque cible ARNm prédite ont été fusionnées à un gène rapporteur *gfp*. Les régions clonées comprennent les domaines d'appariement prédits avec IsrR. Le gène *isrR* a été placé sur un plasmide multicopie, et des mutants dépourvus du premier, du deuxième ou du troisième CRR ont été construits. Les souches  $\Delta isrR$  contenant les différents rapporteurs ont été transformées par un plasmide témoin, ou le plasmide multicopie exprimant IsrR ou ses dérivés. Pour chaque souche contenant l'un des quatre gènes rapporteurs, l'expression d'IsrR a conduit à une fluorescence réduite significative.

L'intégrité du CRR1 était requise pour l'activité d'IsrR contre les fusions rapporteuses *fdhA* et *gltB2*. Cependant, le CRR1 était dispensable pour l'activité d'IsrR contre les fusions rapporteuses *narG* et *nasD*. De façon intéressante, le CRR2 était nécessaire pour l'activité d'IsrR contre les quatre fusions rapporteuses, et l'intégrité d'au moins deux CRR d'IsrR était requise pour l'activité contre les quatre cibles. Ces observations ont révélé que tous les CRR sont nécessaires pour monter la réponse complète d'IsrR, mais seulement certains sont nécessaires pour son activité contre des cibles ARNm données.

**IsrR inhibe le métabolisme des nitrates.** Pour déterminer si IsrR interfère avec le métabolisme du nitrate, la quantité de nitrite dans des cultures anaérobies en présence de nitrate a été mesurée dans des souches exprimant ou non IsrR. Deux de ces systèmes ont été utilisés : 1) une souche  $\Delta isrR$  portant un plasmide IsrR ou un plasmide contrôle et 2) les souches  $\Delta fur$  et  $\Delta fur\Delta isrR$ , où *isrR* est exprimé de manière constitutive dans le contexte  $\Delta fur$ . Dans les deux systèmes, l'accumulation d'IsrR a empêché la production de nitrite, comme prévu si la nitrate réductase (*narG*) est effectivement affectée par IsrR.

**L'activité d'IsrR est indépendante de Hfq.** Chez les entérobactéries, Hfq est un chaperon d'ARN requis pour les régulations médiées par les ARNrég. Cependant, cela ne semble pas être le cas chez *S. aureus* et sa fonction reste énigmatique. Étant donné que l'activité de RyhB est dépendante de Hfq chez *E. coli*, j'ai testé si IsrR nécessitait un Hfq fonctionnel chez *S. aureus*. Les systèmes rapporteurs traductionnels décrits précédemment pour *fdhA*, *nasD*, *narG* et *gltB2* ont été introduits dans des souches  $\Delta hfq$  contenant soit un plasmide témoin, soit un plasmide exprimant constitutivement IsrR. Comme pour la souche parentale, les fusions rapporteuses étaient toujours régulées par IsrR malgré l'absence de Hfq. Cependant, *isrR* cloné sur un plasmide à copies multiples produit des niveaux élevés de ARNrég qui peuvent contourner le besoin de Hfq. J'ai donc introduit le rapporteur de traduction de *fdhA* dans HG003, HG003  $\Delta hfq$  et HG003  $\Delta isrR$ . Lorsqu'IsrR est présent, son expression est induite à partir de son locus

endogène par l'ajout de DIP au milieu. Sur des plaques contenant du DIP, la fusion rapporteuse a montré une activité réduite dans la souche parentale et  $\Delta hfq$  par rapport à la souche dépourvue d'IsrR. De plus, la production de nitrite *in vivo* a été comparée qualitativement entre une souche HG003 WT et son dérivé  $\Delta hfq$  contenant soit un plasmide témoin, soit un plasmide exprimant constitutivement IsrR. Après l'ajout de nitrate ( $\text{NaNO}_3$ ), la souche  $\Delta hfq$  exprimant IsrR a inhibé la production de nitrite comme observé avec la souche parentale. Je conclus que l'activité d'IsrR ne nécessite pas de Hfq pour les phénotypes testés.

**L'ARNrég IsrR est requis pour la virulence de *S. aureus*.** La séquestration du fer par l'hôte joue un rôle crucial pendant l'infection par *S. aureus*. Comme le mutant  $\Delta isrR$  a un défaut d'adaptation dans les environnements restreints en fer, j'ai postulé qu'il pourrait également avoir un impact sur la virulence de *S. aureus*. La virulence d'une souche  $\Delta isrR$ , d'une des souches complémentées avec IsrR et de la souche parentale (HG003) a été comparée lors d'une injection intraveineuse dans un modèle murin d'infection septicémique. La plupart des souris inoculées avec des souches exprimant un IsrR fonctionnel (souches parental et complémentée) sont mortes en 8 jours. À l'opposé, l'absence d'expression d'IsrR a considérablement réduit la virulence de HG003, démontrant le rôle important de cet ARNrég lors de l'infection par *S. aureus* dans ce modèle.

## 4. Discussion

Le lien entre la pathogénie de *S. aureus* et son statut en fer est établi. L'hôte exerce une immunité nutritionnelle qui épuise le fer en réponse aux bactéries envahissantes comme moyen de limiter l'infection. Ici, j'ai identifié IsrR comme le seul ARNrég, sur 45 testés, requis pour une croissance optimale de *S. aureus* dans des conditions de restriction en fer et dont l'absence atténue la virulence de *S. aureus*.

IsrR a déjà été signalé dans a) une étude transcriptomique à grande échelle réalisée dans de nombreuses conditions de croissance, et b) comme l'ARNrég le plus fortement exprimé lorsque la croissance de *S. aureus* en sérum humain (comparée à un milieu de laboratoire riche). Les environnements qui ne fournissent pas de fer libre, comme le sérum, favorisent l'induction de gènes réprimés par le fer. Les découvertes sur la régulation d'*isrR* identifient la privation de fer comme le signal de l'induction d'*isrR*. IsrR est le seul ARNrég testé affecté à la fois par le DIP et l'EDDHA et il est régulé par Fur. L'inactivation du répresseur Fur entraîne une régulation négative

de plusieurs gènes. Cette régulation paradoxale pourrait être obtenue par une expression Fur-dépendante de régulateurs négatifs, qui agissent comme des antagonistes de la réponse Fur, IsrR étant un antagoniste de Fur chez *S. aureus*. Il convient de noter que plusieurs gènes précédemment signalés comme étant réprimés lors d'une privation de fer sont des cibles prédites d'IsrR par CopraRNA, y compris *narG* et *nasD*.

En l'absence d'oxygène, les Staphylocoques utilisent les nitrates et les nitrites comme accepteurs d'électrons de la chaîne respiratoire. Les réductases de nitrate et de nitrite sont exprimées en conditions anaérobies et en présence de nitrate. Ce contrôle est médié par le régulateur transcriptionnel staphylococcique NreABC. IsrR fournit un point de contrôle supplémentaire à la respiration des nitrates en liant la transcription des opérons *narGHJ* et *nirR nasDEF* à la présence de fer, un élément essentiel de ces composants de la chaîne respiratoire.

Lorsque le fer est limitant, IsrR cible divers ARNm exprimant des protéines contenant du fer qui ne sont pas essentielles à la croissance. La voie de réduction des nitrates est inactivée et *S. aureus* peut encore se développer par fermentation dans des conditions anaérobies. Les ARNrég affectant directement le métabolisme de l'azote ont été caractérisés chez les Alphaprotéobactéries, les Gammaprotéobactéries et les Cyanobactéries ; à notre connaissance, IsrR est le premier exemple d'ARNrég du phylum Firmicutes. Dans plusieurs cas, les ARNrég inhibent directement les régulateurs des voies respiratoires de l'azote. L'analyse bioinformatique suggère qu'IsrR pourrait également être l'un d'entre eux en ciblant l'ARNm *nreC*.

Le fer épargné par IsrR peut être redirigé vers des processus vitaux. Par conséquent, IsrR joue un rôle central pour l'adaptation de *S. aureus*, y compris la résistance à l'immunité nutritionnelle de l'hôte. Il est possible que l'inhibition de l'activité d'IsrR par des molécules d'ARN antisens dédiées ou d'autres moyens, s'oppose à la pathogénicité des staphylocoques.

La plupart des ARNrég staphylococciques sont peu conservés entre différentes espèces. Une explication est que les ARNrég agissent principalement par appariement imparfait à des régions non traduites. Contrairement aux ORF (*open reading frames*), ces séquences sont sujettes à des mutations silencieuses et donc à des changements évolutifs rapides. Cependant, *isrR* et sa régulation par Fur sont conservés dans tout le genre *Staphylococcus*. Cette conservation entre les espèces peu courante (un autre exemple chez *S. aureus* est RsaE) révèle une pression sélective pour maintenir intactes la séquence, la structure et la régulation d'IsrR. L'appariement

d'IsrR avec plusieurs cibles d'ARNm liées à des fonctions importantes, telles que l'homéostasie du fer, peut expliquer cette conservation ; la survenue de mutations aléatoires dans *isrR* affecterait le métabolisme lié au fer et serait donc contre-sélectionnée.

Les ARNrég qui inhibent les ARNm codant pour les groupes fer-soufre se trouvent dans les bactéries Gram positives et négatives. En plus d'IsrR, ceux-ci incluent RyhB chez les bactéries entériques, les paralogues PrrF1 et PrrF2 chez *P. aeruginosa*, Mrsl chez *M. tuberculosis*, et FsrA chez *B. subtilis*. Remarquablement, ils ne partagent pas la même dépendance à une protéine chaperon d'ARN (Hfq pour RyhB et PrrF1/PrrF2; FbpA, FbpB et FbpC pour FsrA) avec IsrR, et leurs gènes ne partagent ni synténie ni homologie de séquence. Parmi les exemples discutés, FsrA est le seul ARNrég qui se lie à une séquence Shine-Dalgarno via un motif riche en C. Ces différentes observations indiquent que ces ARNrég ne sont pas des orthologues d'IsrR. Néanmoins, plusieurs ARNm codant pour les mêmes enzymes avec groupes fer-soufre sont des cibles de ces ARNrég. De façon intéressante, la réponse d'épargne du fer de ces ARNrég semble se concentrer sur des cibles impliquées dans les voies respiratoires (métabolisme des nitrates) et les voies métaboliques centrales (cycle du citrate). Concernant ces derniers, l'ARNm codant pour l'aconitase (*acnA/citB*) est ciblé par tous ces ARNrég. Les ARNm codant pour les sous-unités de la succinate déshydrogénase sont ciblés par tous les ARNrég mentionnés ; bien qu'ils ne figurent pas parmi les 23 meilleurs candidats pour IsrR par CopraRNA, les ARNm *sdhA* et *sdhC* sont néanmoins des cibles prédites.

La régulation commune de ces ARNrég par le fer et leurs cibles partagées suggèrent une évolution convergente, qui peut être raisonnablement expliquée comme suit : l'accumulation d'enzymes non essentielles contenant du fer est délétère dans les environnements pauvres en fer ; cependant, un ARNrég induit pendant la carence en fer qui mute pour s'apparier à ces nombreux transcrits dispensables fournit un avantage sélectif immédiat, plus efficace sur le plan énergétique que la production de protéines régulatrices spécialisées. Étant donné que Fur est un répresseur dépendant du fer largement conservé dans les bactéries, tout ARNrég régulé par Fur, provenant éventuellement de transcriptions défectueuses, peut être recruté pour accomplir cette tâche. Les ARN sensibles au fer sont en effet fréquents chez les bactéries. L'évolution à long terme de certains de ces ARNrég devrait conduire à la naissance d'analogues fonctionnels d'IsrR/RyhB. Nos résultats montrent que la régulation basée sur les ARNrég est une stratégie largement conservée et polyvalente pour l'adaptation bactérienne contre la carence en fer, une condition souvent rencontrée dans le cadre de la réponse immunitaire nutritionnelle de l'hôte.

## E. ABBREVIATIONS

Abbreviation	Meaning
BHI	Brain-heart infusion
CFU	Colony forming units
CRR	C-rich regions
DIP	2,2'-dipyridyl
DNA	Deoxyribonucleic acid
EDDHA	ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
GFP	Green fluorescent protein
mRNA	Messenger RNA
OD <sub>600</sub>	Optical density at 600 nm wavelength
ORF	Open reading frame
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RBS	Ribosome-binding site
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SD	Shine-Dalgarno
sRNA	Small RNA
TCA	Tri-carboxylic acid
TCS	Two-component system
TSB	Tryptic soy broth
TSS	Transcription start site
UTR	Untranslated region



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## G. INTRODUCTION

### 1. Iron & bacteria

#### 1.1. Role and properties of iron

Iron is the fourth most abundant element on Earth's surface, and it is critical for the survival of almost all living organisms. Iron exists in two oxidation states, the reduced  $\text{Fe}^{2+}$  ferrous form and the oxidized  $\text{Fe}^{3+}$  ferric form. Under the presence of oxygen, the predominant form of iron is  $\text{Fe}^{3+}$  and it has a very low solubility ( $10^{-18}$  M at pH 7.0), while  $\text{Fe}^{2+}$  is relatively soluble (0.1 M at pH 7.0), which presents a problem for organisms that develop in aerobic environments. Conversely, in anaerobic conditions or microaerobic conditions at low pH, the  $\text{Fe}^{2+}$  is the predominant form.

This transition metal is an extremely versatile electron carrier due to its high variability of  $\text{Fe}^{2+}/\text{Fe}^{3+}$  redox potential, which can be fine-tuned by a wide range of ligands. Its great ability to gain or donate electrons make of iron a very important element in a vast diversity of biochemical reactions and numerous major biological processes such as DNA biosynthesis, TCA cycle,  $\text{N}_2$  fixation, photosynthesis, methanogenesis, respiration, gene regulation,  $\text{H}_2$  production, among many others (1–4).

Moreover, iron can serve as an important co-factor for numerous enzymes, or as a structural prosthetic component incorporating into proteins, either as a mono- or binuclear species, or in a more complex form as part of iron-sulfur (Fe-S) clusters or heme groups.

##### 1.1.1. Iron-sulfur clusters

Fe-S clusters were discovered in the mid-1960's among a group of proteins that contained iron and were involved in biological oxidoreductive processes, the best-known examples being ferredoxins. Since then, Fe-S clusters have been found in all living organisms including archaea, bacteria and eukaryotes, and so far, around 120 different types of proteins are known to contain Fe-S clusters (5,6).

These complexes are polynuclear combinations of iron and sulfur atoms, with the most common clusters being: [2Fe-2S], [3Fe-4S] and [4Fe-4S]. Iron ions are linked to each other through sulfide bridges, and they are bound to the protein by cysteines (Fig. 1).

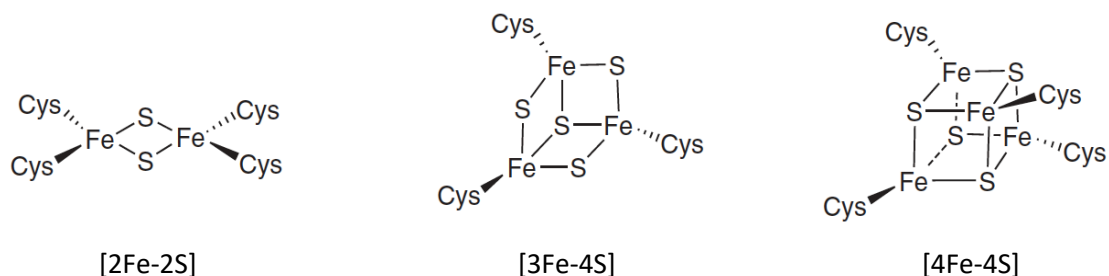


Figure 1. Structure of canonical [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters. Taken from (7).

Fe-S clusters are ideal for electron-transfer and redox reactions due to their extensive redox potential, ranging from more than 500 mV to less than -500 mV, a range larger than any other known redox cofactor. This impressive capacity is in part due to sulfur and iron diverse redox states, but also to the fine-tuning made by different bound ligands, the electronic properties of the protein sites where they are embedded, and potential hydrogen bonds. A given protein can contain a single or multiple Fe-S clusters in a specific spatial arrangement, so electrons can flow efficiently over a long polypeptide chain. While [2Fe-2S] and [3Fe-4S] clusters are usually involved in one-electron-transfer reactions, [4Fe-4S] clusters possess a much diverse chemistry (5,7,8).

Given their exceptional structural plasticity and versatile chemical/electronic features, Fe-S have an active role in electron transfer (as such, Fe-S are important components in the photosynthetic and respiratory electron transport chains), substrate binding and activation, iron/sulfur storage, regulation of gene expression and enzyme activity, sensing of reactive species and sulfur donation (5,7,8).

An excellent review made by Johnson *et al.* (8) summarizes some of the most important biological active roles of Fe-S clusters, which are presented in Table 1 along with some relevant examples. Nevertheless, since Fe-S clusters can be present in almost all kind of proteins, their relevance and length of action as direct/indirect effectors is much wider.

Function	Examples	Cluster type
Electron transfer	Ferredoxins; redox enzymes	[2Fe-2S]; [3Fe-4S]; [4Fe-4S]
Coupled electron/proton transfer	Rieske protein Nitrogenase	[2Fe-2S] [8Fe-7S]
Substrate binding and activation	Dehydratases Radical SAM enzymes Acetyl-CoA synthase Sulfite reductase	[4Fe-4S] [4Fe-4S] Ni-Ni-[4Fe-4S], [Ni-4Fe-5S] [4Fe-4S]-siroheme
Iron storage	Ferredoxins Polyferredoxins	[4Fe-4S] [4Fe-4S]
Structural	Endonuclease III MutY	[4Fe-4S] [4Fe-4S]
Regulation of gene expression	SoxR FNR IRP IscR	[2Fe-2S] [4Fe-4S]/[2Fe-2S] [4Fe-4S] [2Fe-2S]
Regulation of enzyme activity	Glutamine PRPP amidotransferase Ferrochelatase	[4Fe-4S] [2Fe-2S]
Disulfide reduction	Ferredoxin:thioredoxin reductase Heterodisulfide reductase	[4Fe-4S] [4Fe-4S]
Sulfur donor	Biotin synthase	[2Fe-2S]
SAM, S-adenosylmethionine; acetyl-CoA, acetyl coenzymeA; FNR, fumarate and nitrate reduction; IRP, iron-regulatory protein; IscR, iron-sulfur cluster assembly regulatory protein; PRPP, phosphoribosylpyrophosphate.		

Table 1. Examples of functions of some biological [Fe-S] clusters. Taken from (8).

### 1.1.2. Heme iron

Iron protoporphyrin IX, best known as heme, is a molecule consisting of Fe<sup>2+</sup> coordinated within a tetrapyrrole ring; it is the most abundant form of iron in vertebrates. In humans, more than 90% of functional iron in the body is in the form of heme, contained in the protein hemoglobin within erythrocytes. Impressively, each erythrocyte can contain up to 280 millions of hemoglobin molecules, resulting in an iron capacity of around 1 billion atoms per cell (9–11).

Heme enhances iron solubility and catalytic activity after its incorporation as a prosthetic group in proteins called hemoproteins. These enzymes are involved in many essential biological processes such as oxygenation reactions, oxidative stress responses, electron transport, as well

as oxygen-sensing, transport and storage. However, heme is also capable of causing toxicity at high concentrations due to its ability to promote oxidative stress and lipid peroxidation, thus leading to membrane damage and, ultimately, apoptosis; therefore, it is necessary to achieve a tight regulation of its intracellular concentrations (12,13).

In vertebrates, the primary function of heme is to reversibly bind to oxygen ( $O_2$ ) in order to store and transport it across the organism's body. This transport is achieved when the heme- $O_2$  complex is carried by the protein hemoglobin (found in erythrocytes) and at some extent by myoglobin, (found in the skeletal muscle tissue). Hemoglobin transports  $O_2$  from the lungs to several tissues, binding and releasing  $O_2$  in a cooperative manner; nevertheless, heme is also able to bind and transport gaseous molecules such as nitric oxide and carbon monoxide (14–16).

The structural components of heme enable a wide repertoire of interactions with numerous molecules (besides  $O_2$ ) and hemoproteins; furthermore, chemical modifications or substitutions of side chains of the pyrrole rings, result in an even larger number of possible interactions. In general, there are five forms of heme (a, b, c, d and  $d_1$ ) based on the position, structure and type of bonds to various apoproteins. These different variants are presented (Fig. 2), and of these five, heme b is the most common and possesses the basic structure from which the others derive.

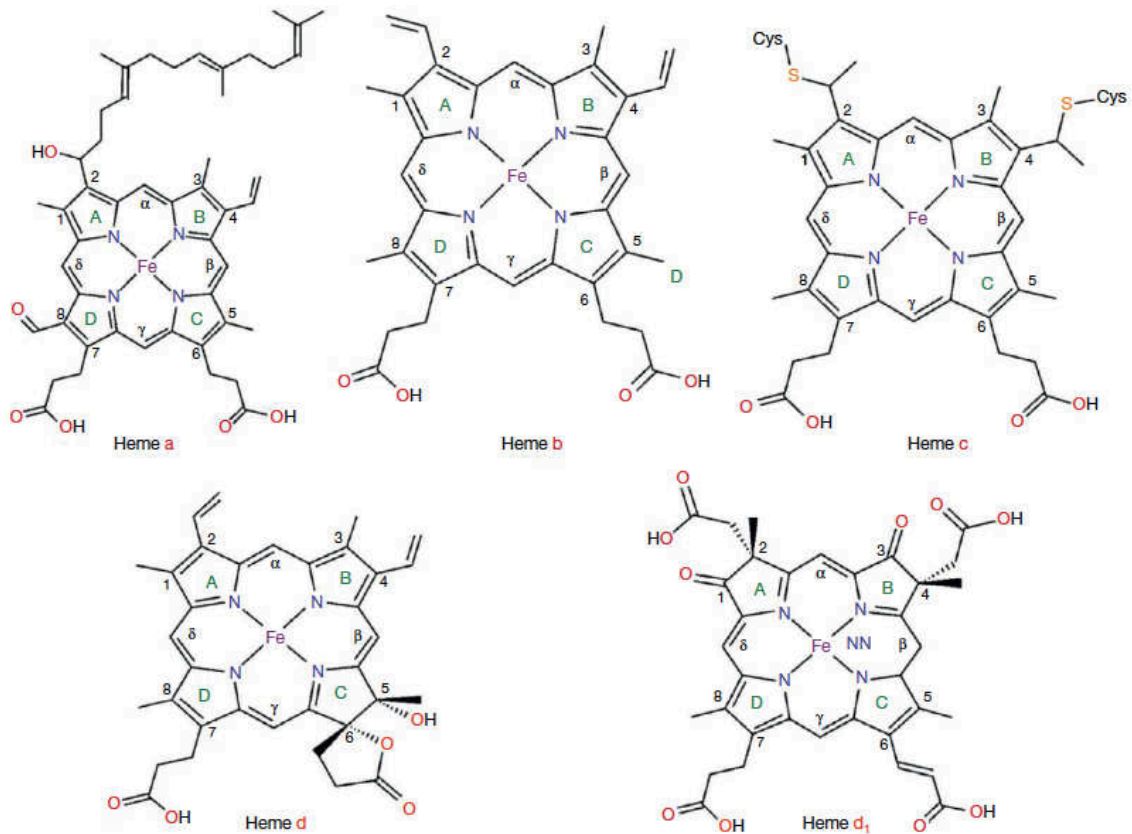


Figure 2. The different forms of heme molecule.  
Taken from (9).

Some examples of functions of heme, either as a cofactor or a prosthetic group within hemoproteins, are presented next (9,12).

- Heme promotes transport, storage or sensing of gaseous molecules through proteins such as hemoglobin, nitrite reductase and NO regulator.
- Heme promotes electron transfer in cytochromes (*e.g.*, cytochrome *c*) within the mitochondria.
- Heme inhibits the activity of mammalian transcriptional repressor Bach 1 and promotes the activity of the Nrf2 activator.
- Heme regulates translation of  $\alpha$  and  $\beta$  globin chains through the heme-sensing inhibitor HRI.

In order to fulfill its iron requirements, all organisms employing this element have developed numerous mechanisms adapted to their specific lifestyle, to obtain a tight balance between iron obtention and its utilization.

### 1.1.3. Iron homeostasis

Interestingly, due to its abundance, prosthetic capability and biocatalyst ability, iron is considered to have been one of the first elements of choice during the development of the first forms of life. Under early oxygen-free stages of earth evolution, iron would have been essential for Eubacteria and Archeobacteria. With the advent of an oxygen-rich environment, the redox properties of iron made it remarkably useful for more complex eukaryotic organisms, which usually possess a metabolism focused on oxygen-based chemistry. Nevertheless, iron also became relatively scarce due to the insolubility of its ferric form, and potentially toxic due to the generation of reactive oxygen species (3–5).

In aerobic environments, the high redox activity of iron can generate through a wide range of mechanisms, free radicals and other strong oxidizing species that can result detrimental for several organisms (especially for prokaryotes). One of the best examples of these mechanisms is the Fenton reaction. This term refers to the reaction between ferrous salts and partially reduced oxygen species like hydrogen peroxide to produce reactive oxygen species (ROS) such as superoxide ( $\text{HO}_2\bullet$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\text{OH}\bullet$ ) (17–19). The overall Fenton reaction is presented below:

1.  $\text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{Fe}^{3+} + \text{OH}\bullet + \text{H}_2\text{O}$
2.  $\text{OH}\bullet + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2\bullet + \text{H}_2\text{O}$
3.  $\text{HO}_2\bullet + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+} + \text{H}^+$

ROS can damage almost all cell components, including membranes, proteins and DNA. The latter being attacked at the deoxyribose backbone or the different bases, resulting in a plethora of lesions which can be cytotoxic or mutagenic (20).

Considering the crucial significance of iron, but also its potential toxicity within most biological systems, it is of vital importance for all living organisms to develop efficient strategies to achieve an adequate iron homeostasis. In this regard, the relevance, as well as the obtention and

regulation of iron will be discussed from now on focusing on bacteria, and especially, on bacteria that can be pathogenic to human beings.

## 1.2. Iron homeostasis in bacteria

Most of essential metal ions for bacteria such as  $Mg^{2+}$ ,  $K^+$ ,  $Zn^{2+}$  and  $Mn^{2+}$  are water-soluble and practically freely available within the host, nevertheless, it is iron the one that possesses more restrictions (21). Most bacteria require iron to survive and proliferate, although there are few exceptions such as some Lactobacilli species (22,23) or the causing agent of Lyme disease, *Borrelia burgdorferi* (24,25).

For the rest of bacteria, the iron content varies for each species, but it generally ranges from  $10^5$  to  $10^6$  atoms per cell depending of growth states and metabolic requirements (26). It has been estimated that at high cell density ( $10^9$  cell  $ml^{-1}$ ) each generation consumes up to  $10^{18}$  iron atoms per liter (27) and indeed, bacteria usually require iron at a concentration of  $10^{-7}$  to  $10^{-5}$  M for optimal growth. Nevertheless, ferric ion has a solubility of  $10^{-18}$  M at pH 7, which translates to just  $6 \times 10^6$  iron atoms per liter resulting in a very low biological availability (4). Of note, some recent studies calculate the solubility of ferric iron as  $10^{-9}$  M at pH 7 (28,29), but even this feature renders very low amounts of bio-available iron for bacterial pathogens.

Bacteria can decrease the gap between iron low-availability and its utilization either by lowering the external pH in order to make ferric iron more soluble; reducing the ferric form to the more soluble ferrous form or by producing iron-scavenging molecules know as siderophores, which also serve as solubilizing agents. The production and mechanism of siderophores will be explained in detail further below.

Non-pathogenic bacteria living in aquatic, terrestrial and even extreme environments can acquire iron from their surroundings, mainly by the production of siderophores and the respective cellular transporters for internalization of the iron-siderophores complexes (30–32).

In the case of pathogenic bacteria, besides the “natural” low availability of iron, their unique lifestyle within the human host presents additional challenges for iron acquisition and utilization since the host will strongly limit the amount of free iron. Therefore, iron acquisition is one of the most crucial determinants during infection, colonization, and prevalence within the host.



### 1.3. Iron availability within the host

In humans, excluding external therapeutic sources, all iron enters the body from the diet mainly in the  $\text{Fe}^{3+}$  form. Dietary iron can be considered either as heme or non-heme iron. Non-heme iron is found in both animal and vegetal sources, being the predominant form in the latter. It can be found as soluble iron, within low molecular weight complexes and in numerous different proteins. The low pH from stomach increases iron solubility and subsequent utilization; moreover, dietary constituents such as citrate and ascorbic acid, and luminal factors can also increase non-heme iron bioavailability. In contrast, heme iron is obtained mainly from animal sources within myoglobin and hemoglobin. Since it is tightly bound to the protoporphyrin ring, heme iron is not susceptible to the mentioned factors acting upon non-heme iron, so, it has generally a lower bioavailability (33,34).

After ingestion from dietary compounds, iron is absorbed by duodenal enterocytes in the small intestine. Non-heme iron is absorbed into the enterocytes via DMT1 in the  $\text{Fe}^{2+}$  form. Since most dietary non-heme iron is in the  $\text{Fe}^{3+}$  form, before its absorption it is reduced to  $\text{Fe}^{2+}$  by the duodenal cytochrome B (35). On the contrary, after its release from the globin by enzymes such as trypsin, heme iron is thought to be absorbed directly through endocytosis of the intact iron-porphyrin complex. Once within the enterocyte,  $\text{Fe}^{2+}$  is released from heme through the action of heme-oxygenases (36,37).

If recently absorbed iron is required, it can be exported into the bloodstream through the FPN1 transporter (ferroportin) (38). But, if recently absorbed iron is not immediately required, it is captured by the iron-storage protein ferritin and conserved within the cell for future use. Ferritin can store large amounts of iron and has ferroxidase activity, converting  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  and promoting mineralization. The core of ferritin can contain around 4000 iron atoms in a mineral form, becoming the major iron storage protein in humans (39).

Within the bloodstream, iron either recently absorbed or released from ferritin storage, is attached to the iron-binding protein called transferrin and it is transported throughout the body to the sites of utilization within various tissues and cells (40). Almost all iron circulating in the plasma is bound to transferrin. Each transferrin molecule can bind up to two  $\text{Fe}^{3+}$  atoms and under normal conditions, only around 30% of the iron-binding sites of the transferrin pool are occupied at a given moment. Transferrin delivers iron into the cells after binding to the

transferrin receptor TfR1 on the membrane. The transferrin-TfR1 complex is internalized by endocytosis; thereafter, the endosome is acidified, the conformation of ferritin changes and  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$ , which lastly, is released from transferrin into the cytoplasm. Once inside the cell, iron can be incorporated into heme, iron-sulfur clusters, and other iron-requiring proteins; or it can be stored within ferritin, creating a storage pool of intracellular iron. Furthermore, transferrin transports iron to the bone marrow, where it serves as an essential element for erythropoiesis (36,41).

Despite fluctuations of iron uptake from diet and intermittent losses from bleeding, the concentration of iron in human plasma remains stable between 10-30  $\mu\text{M}$ . Remarkably, iron absorption, plasma concentration and tissue distribution, are regulated by the hormone hepcidin, which serves as the systemic iron-regulatory hormone (42). The process of iron uptake and transportation through the human body is summarized in Fig. 3.

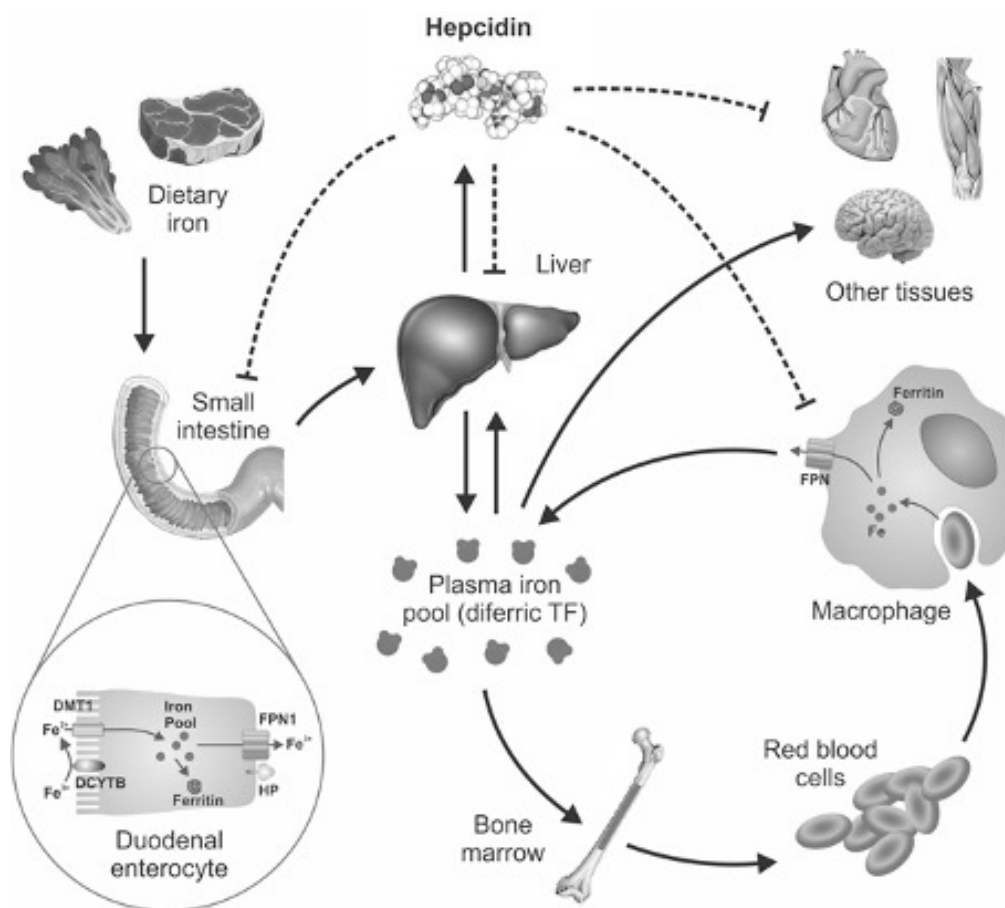


Figure 3. Body iron homeostasis in humans. DCYTB, duodenal cytochrome b; DMT1, divalent metal-ion transporter 1; FPN1, ferroportin; HP, hephaestin; TF, transferrin. Taken from (36).

During normal health conditions, iron homeostasis within the human body is practically constant and adequately regulated. Nevertheless, there exist certain conditions that can disrupt different stages within this process and result in overall disbalance of iron homeostasis.

### 1.3.1. Disorders in human iron homeostasis

Disorders affecting iron homeostasis in humans can result in iron deficiency or iron overload. Iron deficiency can be caused by transient physiological and environmental causes such as insufficient dietary iron, increased demand due to growth, menstruation, pregnancy, drug interference; and some pathologic conditions such as decreased absorption and chronic blood loss. In addition, disruption of any of the different steps of iron homeostasis mentioned above, can also result in iron-deficiency disorders (43,44). Furthermore, a combination of these conditions during a prolonged period of time, can evolve into iron deficiency anemia, which is the best known example of iron-deficiency disorder (45,46).

On the other hand, iron overload can also be caused by numerous factors and generate severe health conditions. Iron-overload disorders can be grouped depending on the underlying pathophysiological defect into three categories (47,48):

- Disorders of the hepcidin-ferroportin axis. The best studied and most common condition is hemochromatosis. This disorder is hereditary and presents elevated transferrin saturation, high serum ferritin and overall tissue iron overload, caused by inadequate or ineffective hepcidin-mediated regulation of the iron transporter ferroportin.
- Disorders of erythroid maturation. This class of disorders includes the so-called iron-loading anemias, which are characterized by an altered erythropoiesis and in some cases, dysregulation of hepcidin production. Examples of these disorders include thalassemias, sideroblastic anemias and dyserythropoietic anemias.
- Disorders of iron transport. In general, these disorders feature an insufficient delivery of transferrin-bound iron for the synthesis of heme. This results in altered erythropoiesis or anemia, which in turn affects hepcidin production and in consequence, iron overload.

Iron overload disorders are of especial interest regarding bacterial infection since iron in excess within the body can become more easily available for pathogenic bacteria and therefore, promote their growth.

Considering that acquisition of vital nutrients such as iron, is a critical step during infection by pathogenic bacteria, the human host possesses several strategies in order to limit the amount of these elements, so they are not available for bacteria. This process is known as nutritional immunity.

#### 1.4. Nutritional immunity

Vertebrate organisms have developed complex defense mechanisms to protect against invading pathogens. A primary line of host defense consists of sequester and starve invading pathogens of vital nutrients such as trace minerals. In 1975, Weinberg coined the term “nutritional immunity” to describe the process by which a host organism sequesters trace minerals in an effort to limit pathogenicity during infection (49,50).

Although nutritional immunity has long been related to host-mediated sequestering of iron, it is currently known that other trace minerals such as zinc and manganese can also be sequestered in order to protect against pathogen invasion (51,52).

The human host exerts several nutritional immunity responses to withhold iron from bacterial pathogens. Some examples are presented below.

- Infection and the consequential inflammation stimuli induce hepcidin production, which in turn promote the degradation of the iron exporter, ferroportin. This results in inhibition of iron transfer into the bloodstream from enterocytes, macrophages, hepatocytes and other iron-storing cells. This process can decrease serum iron levels up to 30% and is usually referred as the “hypoferremia of infection” (50,53).
- The vast majority of iron is sequestered intracellularly, strongly complexed to heme and incorporated either into hemoglobin or myoglobin (13,54).

- Host plasma glycoproteins haptoglobin and hemopexin, scavenge and bind with very high affinity to free hemoglobin and heme, respectively. Once hemoglobin and heme are retrieved, they are transported to the liver for iron-recycling (55,56).
- Extracellular iron is bound with high affinity by transferrin. Normal levels of unsaturated transferrin help to maintain the amount of free iron in tissue fluids at levels that are restrictive for bacterial growth (57,58).
- Iron is mainly stored within ferritin, which is located mostly in the cytoplasm but also in the mitochondria or the circulating plasma. Besides its iron-storage activity, ferritin possesses antioxidant functions (39,59).
- During infection, neutrophils in serum secrete lactoferrin, a glycoprotein structurally related to ferritin, which scavenges and binds extracellular iron. Lactoferrin is mostly found in the lymph and mucosal secretions and is a key regulator of host's inflammatory and immune responses (60,61).
- Host down-regulates iron transporters such as NRAMP1, which is located in the phagosomal membrane of lysosomes and phagosomes within monocytes, macrophages and T lymphocytes. Inhibition of iron export into the cytosol, limits iron availability for intracellular pathogens (62,63).
- Production of siderocalins, which are proteins that bind and inactivate specific siderophores produced by determined bacterial pathogens (64,65).

Although these mechanisms aim to withhold iron during infection, pathogenic bacteria have evolved numerous strategies to overcome them and obtain the iron required for their survival and proliferation. This permanent battle for iron between the host and bacteria has been the focus of numerous studies over the years in many different pathogenic species. Next, the bacterial strategies for iron acquisition will be discussed.

## 1.5. Iron acquisition in pathogenic bacteria

While some of the iron-acquisition mechanisms are highly conserved and widely employed by many different commensal and pathogenic bacteria, some others are highly specific and closely related to the unique pathophysiology and iron requirements of a determined bacterium.

Broadly, the most common mechanisms of iron acquisition include a) extraction and uptake of heme-iron contained in host hemoproteins through the use of secreted factors or cell membrane-associated receptors; b) secretion of siderophores for the acquisition of free iron in the environment or iron bound either to transferrin or lactoferrin, and its subsequent uptake through specialized transporters; c) uptake of free inorganic iron facilitated by ferric iron reductases and ferrous iron permeases (66). The details of these mechanisms and their impact in the virulence of bacterial pathogens are discussed next.

### 1.5.1. Acquisition of heme-iron

Heme is a particularly attractive target for iron acquisition since it represents the most abundant reservoir of functional iron within the host. Furthermore, it is the preferred iron source for heme-auxotrophic bacteria, which are bacteria unable to synthesize heme so they must obtain it from external sources. Some examples of heme-auxotrophs are staphylococcal small colony variants, *Haemophilus influenzae*, *Enterococcus faecalis*, *Streptococcus agalactiae*, *Bacteriodes fragilis*, among others (67,68).

Since heme exists mostly in strong association with hemoglobin within circulating erythrocytes, invading pathogens have developed intricate mechanisms to access heme contained in cellular hemoproteins. The secretion of hemolysins is a strategy widely employed by pathogens to lyse erythrocytes and enhance heme availability. Indeed, it has been observed that the production of hemolysins is an important determinant for bacterial virulence and pathogenicity (69).

Once erythrocytes are lysed and hemoglobin freed, bacteria can either release heme-scavenging proteins known as hemophores and carry it to cell-surface receptors or deliver hemoglobin (as well as other hemoproteins) to the cell surface for direct uptake through specific transporters (70).

Hemophores are able to capture both free heme and heme bound to hemoproteins (71). Until now, two major types of hemophores have been characterized. In Gram-negative pathogens, HasA-type hemophores deliver heme to the TonB-dependent transporters on the outer membrane, such as HasR, and afterwards to ATP-binding cassette (ABC) transporters on the inner membrane (72,73). However in Gram-positive pathogens, NEAT-domain containing hemophores deliver heme through complex mechanisms of transport across the peptidoglycan cell wall (74). From these mechanisms, the Isd pathway is the best known and it has been extensively investigated in *Staphylococcus aureus*; it will be described later in detail.

For both Gram-negative and positive bacteria, once the heme-iron complex has been internalized, iron can either be released through the oxidative degradation of heme via proteins called heme-oxygenases (75) or heme-iron can be used intact as a cofactor in catalases, cytochromes and other bacterial hemoproteins (Fig. 4). Furthermore, since heme is toxic at high concentrations, bacteria possess tightly regulated systems dedicated to control heme import and detoxification (76). Some examples of bacterial heme acquisition systems that are essential for virulence are presented (Table 2).

Bacterium	Heme acquisition system
<i>Staphylococcus aureus</i>	Isd
<i>Bacillus anthracis</i>	Isd
<i>Bordetella pertussis</i>	Bhu
<i>Escherichia coli</i>	Chu
<i>Haemophilus influenzae</i>	Hbp
<i>Listeria monocytogenes</i>	Hup
<i>Neisseria meningitidis</i>	HmbR
<i>Streptococcus pyogenes</i>	Shr

Table 2. Examples of heme acquisition systems in pathogenic bacteria. Taken from (66).

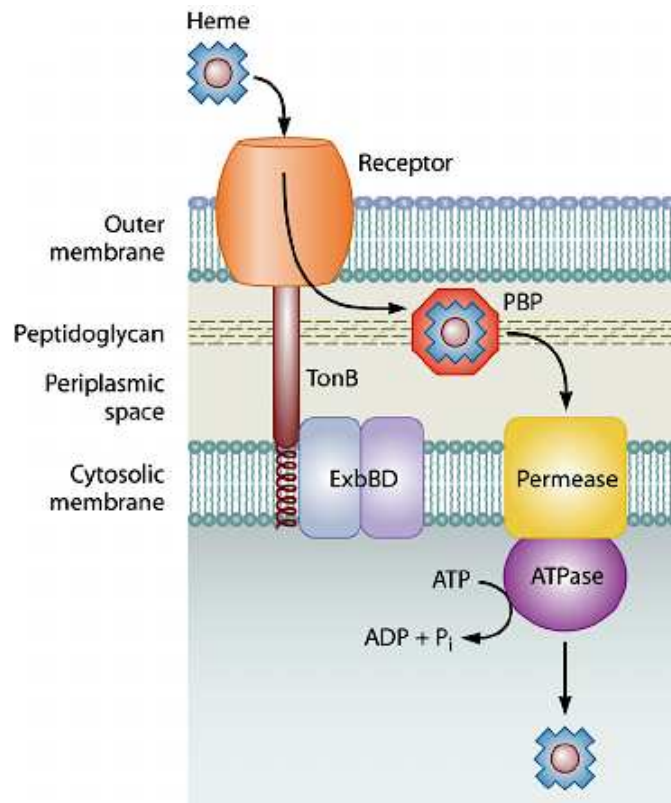


Figure 4. Heme acquisition in Gram-negative bacteria.

Heme binds to a receptor in the outer membrane that transport it into the periplasm using energy transduced from the TonB/ExbBD complex. A periplasmic binding protein (PBP) transfers the heme to a permease in the inner membrane and it is transported into the cytoplasm mediated by an ATPase. Taken from (77).

### 1.5.2. Iron acquisition via siderophores

Siderophores are natural iron chelating molecules of low molecular weight (~400-2000 Da) and very strong affinity for ferric iron. Siderophores are biosynthetically produced and secreted by many bacteria, fungi, and plants during iron starvation with the objective of scavenge iron from the environment and form soluble iron complexes for their uptake and utilization. There exist over 500 known siderophores so far and new structures are being discovered continuously (78–80).

In bacteria, the use of siderophores is the most common mechanism for iron acquisition, especially for pathogenic bacteria that face extremely low amounts of available iron within the human host. Interestingly, siderophores are among the strongest iron-binding agents known. Bacterial siderophore affinities to iron are usually higher than those of host hemoproteins. For example, binding stability of iron to human transferrin (represented by the constant  $K_S$ ) is around



$10^{20}$ , while bacterial siderophores  $K_s$  values are between  $10^{22}$  to  $10^{50}$ , therefore, they are able to sequester iron from such hemoprotein. On the other hand, hemoglobin is not prone to siderophore effects, since it contains iron in the  $\text{Fe}^{2+}$  and siderophores bind exclusively to  $\text{Fe}^{3+}$  (21).

The structure of siderophores consists of a peptide backbone, with modified amino acid side chains that create different iron-coordinating ligands, preferentially forming a hexacoordinated complex with oxygen binding to  $\text{Fe}^{3+}$ . Considering these ligands, siderophores can be classified broadly into three main groups: catecholate, hydroxamate and carboxylate siderophores (Fig. 5). A fourth group of siderophores has usually been designed as “mixed” since they employ more than one of the aforementioned types of moieties (66,81).

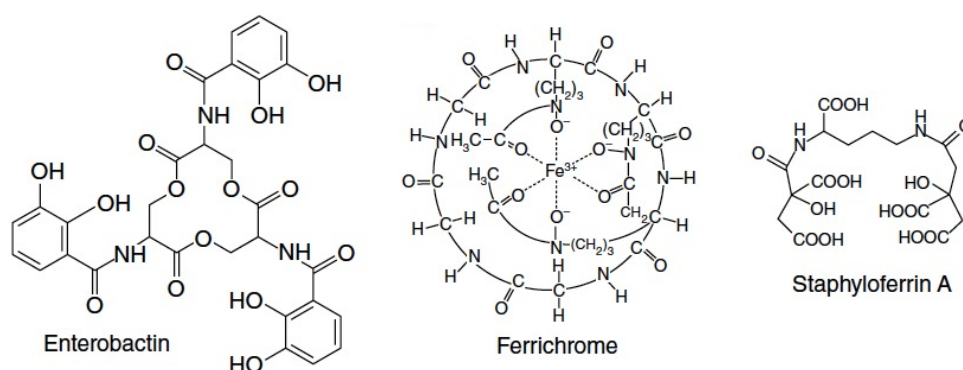


Figure 5. Examples of representative bacterial siderophores. Enterobactin (catecholate), produced by *Escherichia coli*; ferrichrome (hydroxamate), produced by *Pseudomonas aeruginosa*; and staphyloferrin A (carboxylate), produced by *S. aureus*. Note that the same siderophore can be produced by several different species (82). Taken from (83).

Although not discussed here in detail, siderophores are synthesized in the cytoplasm using either a multimodular enzyme scaffold referred to as non-ribosomal peptide synthetases (NRPS), which resembles antibiotic synthesis machinery, or NRPS-independent systems. Genes encoding the enzymes for siderophore synthesis are iron regulated and often located nearby genes involved in siderophore uptake (84,85). Once synthesized, siderophores are exported from the cytoplasm to the extracellular milieu, and although this mechanism is not completely understood yet, it is thought that this process is mediated by membrane transporters of the major facilitator superfamily (86) and subsequently across the outer membrane in Gram-negative through multidomain efflux pumps or ABC-type family transporters (87).

After siderophores find and attach to extracellular  $\text{Fe}^{3+}$ , they are imported back across the cell membrane. Iron transport across the membrane is an active process and requires complex systems in both Gram-negative and Gram-positive.

Among Gram-negative bacteria, siderophores are recognized and transported across the outer membrane by specific TonB-dependent transmembrane receptors such as FhuA in *E. coli*, which is the transporter for the siderophore ferrichrome and possesses the basic structure for many other TonB-dependent receptors (*e.g.*, FepA for enterobactin and FecA for ferric citrate). The transport process requires energy in the form of protonmotive force that is facilitated by a complex of three proteins anchored in the cytoplasmic membrane, TonB-ExbB-ExbD, designated as the TonB system (88,89).

Once in the periplasm, siderophores are bound to specific periplasmic binding proteins (PBPs), such as FhuD in *E. coli* (which mediates the uptake of several hydroxamate-type siderophore), and carried to the cytosolic membrane where they are delivered to a specific ABC transporters for subsequent transport into the cytoplasm (90).

Upon delivery of the iron-bound siderophore into the cytoplasm, iron is reduced from  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by a ferric reductase and subsequently released from the siderophore, due to a strongly decreased affinity and binding-strength. After being released, iron is available either for immediate use by the bacteria or intracellular storage for future use. Meanwhile siderophores are usually left intact for its re-utilization (83) (Fig. 6).

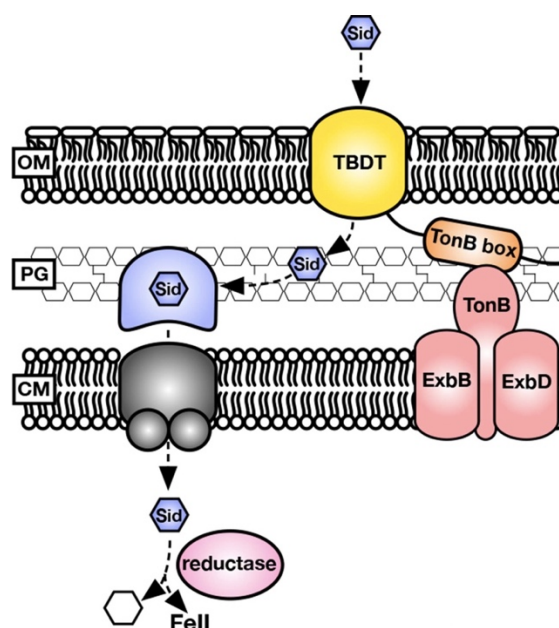


Figure 6. Mechanism of siderophore uptake in Gram-negative bacteria.

Iron-loaded siderophore (blue hexagon) is recognized by a specific TonB-dependent transporter (TBDT). Employing energy provided by proton motive force of the TonB system, the siderophore is transported across the outer membrane (OT) into the periplasm (PG, peptidoglycan), where it is bound to a periplasmic binding protein that carries it to an ABC-transporter within the cytoplasmic membrane (CM). Iron is reduced by a reductase, released from the siderophore, and delivered into the cytoplasm. Modified from (66).

In the case of Gram-positive bacteria, TonB-dependent receptors as well as the TonB system are absent since they lack an outer membrane and a periplasm. Instead they possess lipoproteins attached to the cell wall that recognize the siderophore-iron complex and deliver it to the respective ABC transporter (91,92) (Fig. 7).

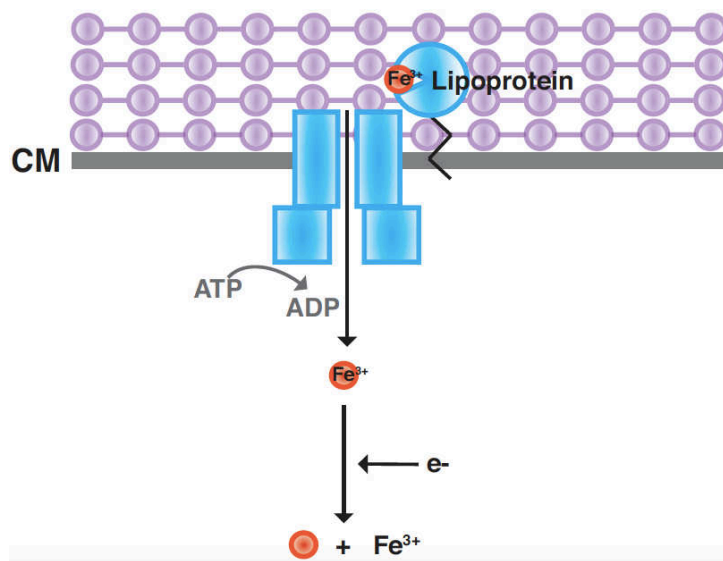


Figure 7. Mechanism of siderophore uptake in Gram-positive bacteria.

Due to the absence of an outer membrane and a periplasmic space, iron-loaded siderophores are recognized by specific membrane-anchored binding proteins and transported into the cytoplasm by a membrane-associated ABC transporter. Subsequently, iron is reduced and released from the siderophore for its use within the cell. Taken from (93).

Most siderophore receptors are highly specific, nevertheless, some possess broad substrate specificity and are able to accept different types of siderophores, and in some cases, even those produced by other bacterial species, which are known as “xenosiderophores”. Bacteria capable to acquire xenosiderophores may have a competitive advantage in polymicrobial communities, increasing their chances to use the few available iron from the environment and without the energetic demands of synthesizing siderophores *de novo* (81).

### 1.5.3. Iron acquisition from transferrin

Some pathogenic bacteria have developed strategies to obtain iron from host iron-binding glycoproteins transferrin and lactoferrin. Two main mechanisms for this obtention are known so far: employment of siderophores and production of cell surface-associated transferrin- or lactoferrin-binding proteins. While these two mechanisms are not mutually exclusive, many enteric pathogens rely on the former, while several mucosal pathogens rely on the latter. This is due in part because the aforementioned glycoproteins can be found in many mucosal surfaces of the upper respiratory tract and the urogenital tract, where they become one of the most readily available iron sources (94).

As example, members of the families *Neisseriaceae* and *Moraxellaceae*, which colonize mainly mucosal surfaces, are capable of using transferrin and lactoferrin as iron sources, without producing a siderophore intermediate. Moreover, some pathogenic *Neisseria* species, besides using these glycoproteins directly, possess receptors for xenosiderophores containing iron obtained from transferrin molecules (88,95,96).

In Gram-negative bacteria, the system for iron removal and uptake from transferrin is composed of the outer membrane receptor protein TbpA, and a surface-associated lipoprotein, TbpB (transferrin-binding protein A and B, respectively). While TbpA can bind to both apo- and holo-transferrin, TbpB binds only to holo-transferrin and it is thought to serve as bait to acquire iron-bound transferrin and deliver it to TbpA for TonB-dependent uptake. In contrast to siderophores and heme, which are transported intact through the outer membrane, iron must be first released from transferrin prior its uptake. This is achieved by conformational changes in holo-transferrin induced by TbpA, which alter iron binding and thus facilitate its release and immediate uptake (97,98).

In addition to iron uptake from transferrin, some pathogenic *Neisseriaceae* and *Moraxellaceae* species are capable of obtaining iron from lactoferrin using an analogous system composed of LbpA and LbpB (lactoferrin-binding protein A and B, respectively), which in general terms, functions in the same way as TbpA and TbpB (99).

Furthermore, once that  $Fe^{3+}$  passes across the outer membrane it can be bound to FbpA (ferric-iron-binding protein A), which is structurally similar to eukaryotic transferrin and functions in a

similar way as a bacterial transferrin, carrying iron across the periplasm to finally deliver it to the FbpBC transporter for its uptake into the cytoplasm (97,100) (Fig. 8).

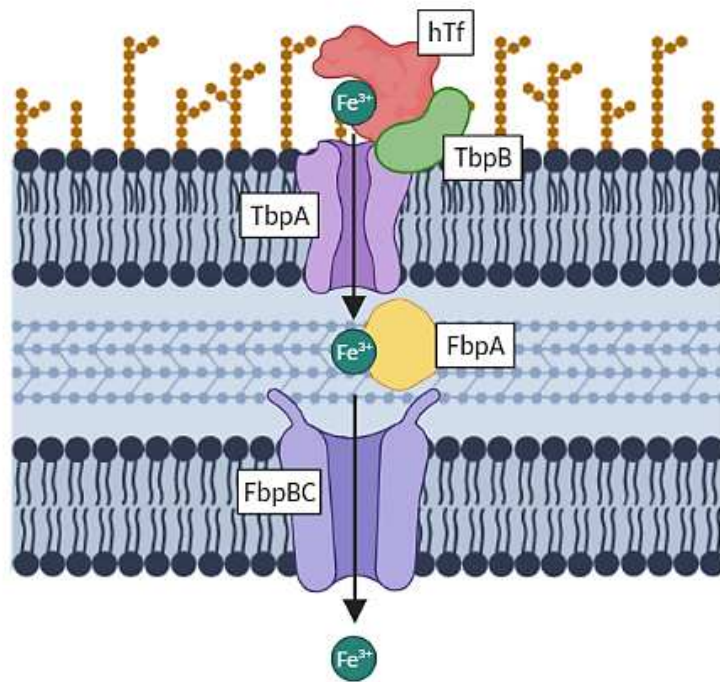


Figure 8. Iron acquisition from human transferrin in Gram-negative bacteria. Iron-loaded human transferrin (hTf) binds to TbpB and it is then delivered to TbpA for TonB-dependent transport. Conformational changes in holo-transferrin induced by TbpA alter iron binding and promote its release. Once that iron enters the periplasm, it is bound to FbpA which delivers it to the FbpBC transporter for its uptake into the cytoplasm.

#### 1.5.4. Acquisition of inorganic iron

Since iron acquisition from heme and through siderophores are the best-known mechanisms for its obtention, and considering the relatively low availability of inorganic iron, bacterial systems for acquisition of free iron have been sparsely characterized.

In anoxic and low pH conditions (*e.g.*, within the human gut), iron can be converted from its ferric insoluble form to the more soluble ferrous ( $\text{Fe}^{2+}$ ) form and it can be imported directly by high affinity ferrous iron transporters. In Gram-negative bacteria, it is thought that ferrous iron can diffuse freely through outer membrane porins and once in the periplasm, it is transported actively into the cytoplasm via different systems such as MntH, EfeUOB and Feo. Of these, the latter is the best studied inorganic iron acquisition system in bacteria (93).

The ferrous iron transporter system (Feo) is widely conserved in both Gram-positive and Gram-negative bacteria, although it has been mainly characterized in members of the *Enterobacteriaceae* family such as pathogenic *E. coli*, *Shigella* spp., *Salmonella* spp., and *Yersinia pestis*. This system is composed of three proteins: FeoA, a small cytoplasmic protein; FeoB, a large transmembrane protein; and FeoC, a second small cytoplasmic protein present only in some  $\gamma$ -proteobacteria, nevertheless, the actual mechanism of how this system functions at the molecular level remains to be elucidated. The function of FeoA remains unknown and FeoC is thought to act as an [Fe-S]-dependent transcriptional regulator of the *feoABC* operon, although this has not been confirmed. However, FeoB has been better characterized and it has been found as the only essential protein of the Feo system (101).

FeoB protein is embedded in the inner-membrane and likely functions as the  $\text{Fe}^{2+}$  permease. Iron transport through FeoB requires binding of GTP to a G protein domain located in the N-terminal cytoplasmic region (NFeoB), which apparently has a GTPase activity and provides the energy necessary for iron translocation. Alternatively, the state of GTP bound to NFeoB could regulate the opening and closing of the Feo protein (102) (Fig. 9).

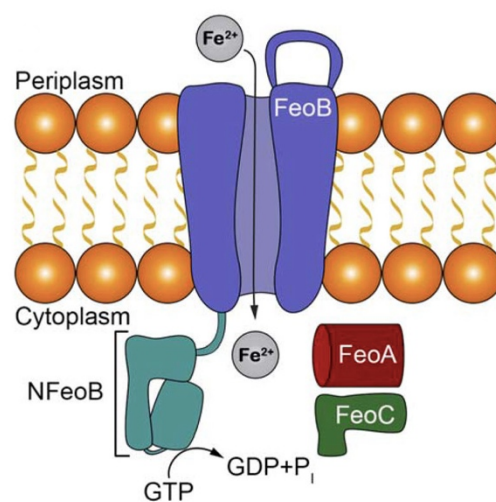


Figure 9. Feo-mediated ferrous iron transport. Ferrous iron ( $\text{Fe}^{2+}$ ) moves through membrane bound FeoB transporter from the periplasm into the cytosol. GTP hydrolysis by NFeoB serves as an energy source for this transport process. Function of FeoA and FeoC within this process remain unknown. Taken from (102).

The expression of the *feoABC* operon is regulated by the transcriptional factor Fnr (in response to oxygen deprivation) and, as many other systems involved in bacterial iron homeostasis, by the iron-sensitive ferric uptake regulator, Fur (93,101,102).



A summary of the different iron acquisition mechanisms in bacteria is presented (Fig. 10).

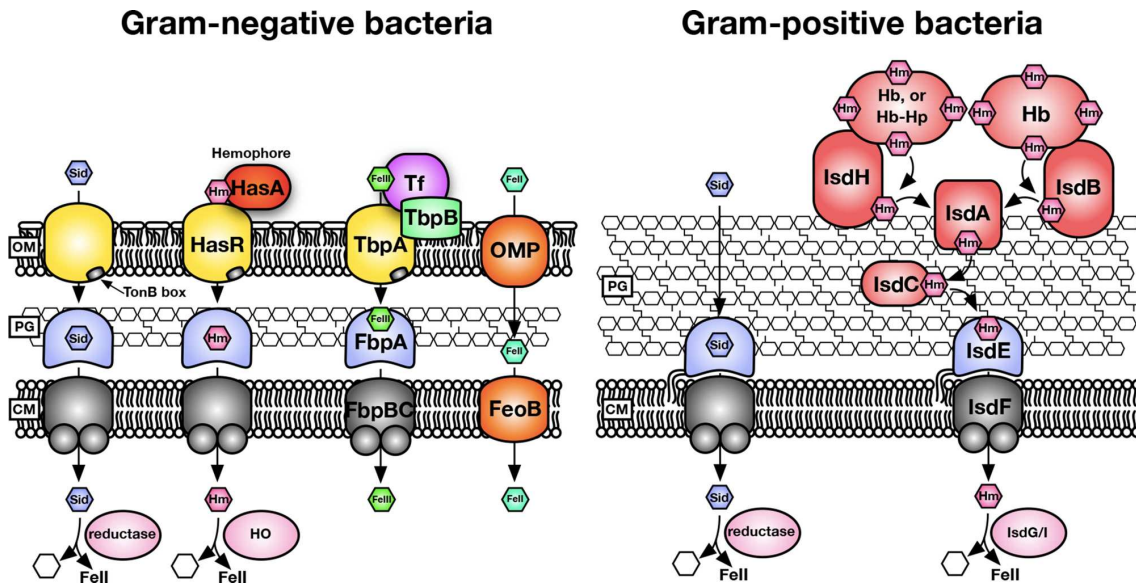


Figure 10. Mechanisms of iron acquisition in Gram-negative and Gram-positive bacteria. Diagrams depicting the envelope proteins required for the uptake of iron, or iron scavenged from siderophores, heme, or transferrin. This is a composite diagram and represents mechanisms used by many pathogenic bacteria, as described in the text. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; sid, FeIII-siderophore; Hm, heme; Tf, transferrin; OMP, outer membrane porin; HO, heme oxygenase; Hb, hemoglobin; Hp, haptoglobin. Taken from (66).

## 1.6. Ferric uptake regulator

In bacteria, the expression of many genes involved in nutrient acquisition is regulated by transcriptional factors in response to specific environmental signals. In the case of iron acquisition, it is well known that Fur, the ferric uptake regulator, is one of the most important elements involved in this process, and consequently, a major contributor to bacterial iron homeostasis.

Fur is a DNA-binding protein that uses  $Fe^{2+}$  as a co-repressor and inhibits the transcription of genes mainly involved in iron acquisition after binding to a specific DNA motif in their promoter region and blocking the entry of RNA polymerase. In absence of  $Fe^{2+}$ , Fur is inactive (103) (Fig. 11).

Fur is conserved in many Gram-negative and positive bacteria (103,104). It was initially described in *E. coli* and *S. typhimurium*, where it represses transcription of genes involved in siderophore synthesis and transport. It was observed that *fur* mutants expressed constitutively

siderophores and their membrane receptors (105,106). Subsequent analysis of the promoter region of different Fur-regulated genes, defined the  $\approx 19$ nt consensus sequence recognized by this protein, GATAATGATAATCATTATC, known as the Fur box (107,108). Additionally, it has been shown that active  $\text{Fe}^{2+}$ -bound Fur acts as a homodimer (109).

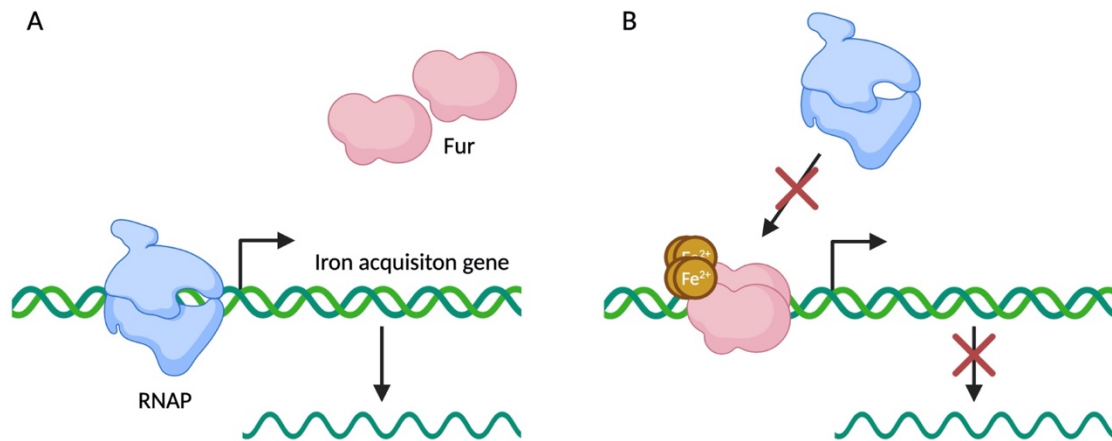


Figure 11. Classic model of Fur repression.

A) Under low iron conditions, Fur is inactive and transcription of iron acquisition genes by RNA-polymerase (RNAP) can proceed. B) When iron is abundant, it binds to Fur, which in turn inhibits the transcription of iron acquisition genes after binding to the Fur box in their promoter region and blocking the entry of RNA-polymerase.

The Fur protein is composed of an amino-terminal domain and a carboxy-terminal domain, the latter containing the binding site for  $\text{Fe}^{2+}$ . Nevertheless, it has been shown that other divalent cations such as  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  can also bind at different rates (110). Moreover, Fur belongs to a superfamily of transcriptional regulators that use these divalent cations for activity, and which are involved mainly in bacterial metal homeostasis (111). Among Fur superfamily, PerR (peroxide stress response) and Zur (zinc uptake regulator) are some of the best-studied members, and together with Fur, they can act simultaneously in several regulatory mechanisms (112,113). While PerR mostly regulates oxidative stress-protecting proteins and iron-storage proteins (114), Zur regulates proteins involved in zinc metabolism (115).

The Fur regulon is vast. For example, in *E. coli* more than 80 genes have been found to be regulated by Fur (116–118). Nevertheless, the Fur regulon is constantly expanding, and it varies widely according to each bacterial species. Besides its fundamental role in iron homeostasis (siderophore production, iron transport and storage, etc.), Fur has an important role in diverse processes such as oxidative stress response, DNA synthesis, energy metabolism and biofilm formation, among many others (117,119) (Fig. 12).



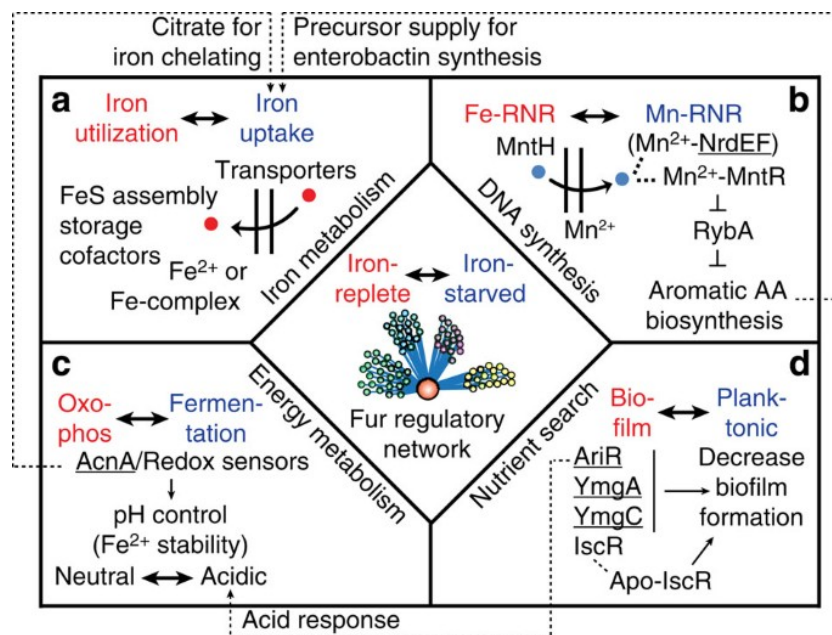


Figure 12. Global coordination roles of the Fur regulatory network in *E. coli*. Fur directly regulates genes associated with a) iron metabolism, b) DNA synthesis, c) redirection of metabolism towards fermentative pathways and d) biofilm formation for searching nutrients in response to iron availability. These networks are linked through Fur regulation. Taken from (117).

Fur has an important role in many bacterial cellular processes including a significant effect on virulence in different pathogenic bacteria (104,120). One of the best examples is its protection role against host ROS. Since most superoxide dismutases and  $H_2O_2$ -degrading enzymes (e.g. SodA in *E. coli* or KatA in *S. aureus* (121,122)) require divalent metal cofactors for enzymatic function, and because Fur is itself a redox sensing protein, it exerts an active role in the regulation of bacterial defenses against ROS (123,124).

Although Fur is mainly a  $Fe^{2+}$ -dependent repressor, there are cases where Fur is able to directly activate gene expression. For example, it has been shown that transcription of *norB* in *Neisseria meningitidis*, *hilD* in *Salmonella typhimurium* and *oorB* in *Helicobacter pylori*, is activated by Fur. In these cases, the Fur-box is located around 100-200 nt upstream of the transcriptional start site, and although the exact mechanism of activation is unknown, it has been shown that Fur enhances recruitment of RNA polymerase to the promoter region of these genes (125–127) (Fig. 13).

Additionally, Fur can activate transcription working as an “anti-repressor”. In *E. coli*, the histone-like protein H-NS binds to the promoter region of *ftnA*, the gene encoding the iron-storage protein ferritin (pg. 15) and inhibits its transcription. In iron replete conditions, Fur binds

upstream the promoter region of *ftnA*, blocking the entry of H-NS and consequently allowing transcription of *ftnA* and overall promoting iron storage (128) (Fig. 13).

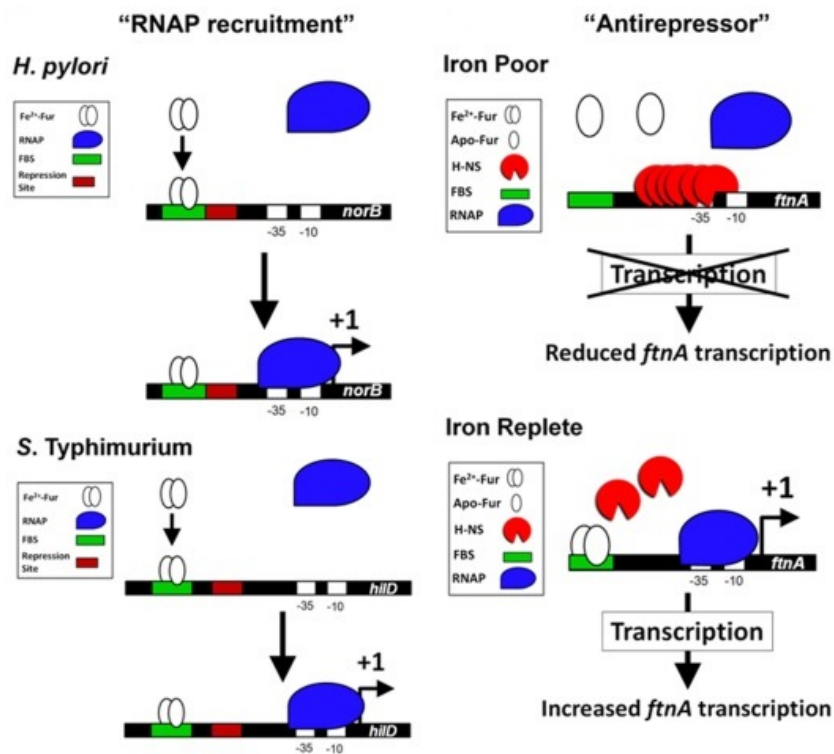


Figure 13. Mechanisms of Fur activation.

Left panel: Fur activation through “RNAP recruitment” mechanism. For *S. Typhimurium* and *H. pylori* active Fur-Fe<sup>2+</sup> binds to a Fur-binding site (FBS) promoting recruitment of RNAP and subsequent transcription of *norB* and *hlyD*, respectively. Right panel: Fur activation through “antirepressor” mechanism. In *E. coli*, under low iron conditions H-NS binds upstream *ftnA* gene and inhibits its transcription. When Fur is active, its binds to the promoter region of *ftnA* preventing H-NS binding and even physically removing it, allowing *ftnA* transcription. Modified from (103).

Besides these known mechanisms of direct transcription activation by Fur, for a long time, a paradigmatic mechanism of “positive regulation” by Fur remained unexplained. It was suggested that in *E. coli*, the expression of the superoxide dismutase gene *sodB* was activated by Fur, however, no putative Fur boxes were identified in the promoter region of *sodB* (129). Nevertheless, it was observed that Fur-mediated activation of *sodB* was iron-dependent and additionally, that *sodB* mRNA half-life greatly decreased in a  $\Delta fur$  mutant (130).

Additionally, earlier studies demonstrated that  $\Delta fur$  mutants could not grow on succinate or fumarate as a sole carbon source, demonstrating a fitness impairment probably due to the downregulation of some elements involved in the metabolism of these compounds (131,132).

Direct iron-dependent regulation by Fur has been demonstrated as a crucial mechanism in iron homeostasis in bacteria. Nevertheless, 20 years ago, a Fur-regulated non-coding RNA named RyhB emerged as another outstanding element within bacterial iron homeostasis, and its regulatory effects explained the Fur paradigm (see further below).

While transcriptional factors such as Fur exert an important regulation in many cellular processes in response to specific environmental conditions, over the last decades, the role of non-coding RNAs as additional global regulators have become increasingly significant, after the demonstration of their importance in several regulatory networks among different bacteria.

## 2. Non-coding regulatory RNAs

Bacterial non-coding RNAs are a diverse group of molecules that despite not being translated into a protein, act by numerous mechanisms to modulate a wide range of cellular processes.

These regulatory RNAs were discovered in the 1980's (133–135), years before the discovery of microRNAs (miRNAs) and short interfering RNAs (siRNAs) in eukaryotes. Since then, technical advances, including multilayered computational searches, next-generation sequencing, and tiled microarrays with complete genome coverage, have enabled the identification of new regulatory RNAs in many different bacterial species. For example, in *E. coli*, around 80 regulatory RNAs have been verified to date, increasing the total number of genes by 2% in this bacterium (136). Identification and description of regulatory RNAs in the recent years highlighted their important role in bacterial physiology.

In overall, bacterial regulatory RNAs can modulate transcription, translation or mRNA stability, and they achieve these outcomes through numerous mechanisms such as modification of RNA structure, protein binding, base-pairing with other RNAs, among others (137).

There exist several kinds of bacterial regulatory RNAs, and according to their mechanism of action they can be classified either as *cis*- or *trans*-acting RNAs.

## 2.1. *Cis*-acting regulatory RNAs

The best-known example of *cis*-acting RNAs are riboswitches, which are sequences usually located in the 5'UTR of the mRNA they regulate. In response to environmental signals, riboswitches can undergo conformational changes that lead to changes in the expression of the downstream genes (138,139). Despite of their importance in bacterial physiology, *cis*-acting RNAs will be no further discussed.

## 2.2. *Trans*-acting regulatory RNAs

On the other hand, *trans*-acting RNAs can exert their regulatory functions in a different place from where they were expressed, binding either to proteins or to other RNAs and modulating their activity. Additionally, these regulatory RNAs have usually a small size, ranging from around 50 to 500 nucleotides. For this reason, bacterial non-coding regulatory RNAs are commonly referred as sRNAs (small RNAs) (140,141), which is the chosen name to describe them from now on.

Of note, there are some exceptions to the criteria mentioned above. One of the best-known examples is RNAIII in *Staphylococcus aureus*, which is a regulatory RNA with an important role in quorum sensing, stress response, virulence, *etc.*, but also encodes the  $\delta$ -hemolysin Hld and has an unusual large size of 514 nucleotides (142,143).

sRNAs that bind proteins (beside RNA chaperones) belong to a so far small group of regulatory RNAs. Among the best-studied examples are tmRNA, CsrB, 6S and GlmY in *E. coli*.

Transfer-messenger RNA (tmRNA) is a bifunctional RNA that has properties of a tRNA and an mRNA, and it is highly conserved among bacteria. tmRNA acts as a quality control system that monitors protein synthesis and recycles stalled translation complexes in a process termed "ribosome rescue". During rescue, tmRNA acts first as a tRNA and binds to stalled ribosomes, then as a mRNA to add the *ssrA* peptide tag to the C-terminus of the nascent polypeptide chain. *ssrA*-tagged proteins are rapidly degraded by proteases, ensuring rapid degradation of potentially deleterious truncated polypeptides. Ribosome rescue also facilitates turnover of the damaged messages responsible for translational arrest (144,145).

CsrB binds to CsrA protein, which regulates mRNAs of genes involved in biofilm formation, motility, virulence, quorum sensing, stress response, among others. Sequestration of CsrA by CsrB results in altered translation and/or turnover of its targets (146,147). On the other hand, 6S RNA is a regulator of RNA polymerase and it is broadly conserved among bacteria. During transcription initiation, 6S RNA mimics the “open complex” conformation of promoter DNA and binds to housekeeping holoenzyme form of RNA polymerase ( $\sigma^{70}$ -RNAP), resulting in regulation of transcription. Consequently, transcription of numerous  $\sigma^{70}$ -regulated genes is altered, especially during late stationary phase, when 6S RNA levels are maximal. Occupation of  $\sigma^{70}$ -RNAP by 6S RNA forces the cell to use alternative  $\sigma$  factors such as  $\sigma^5$ , which is responsible of the expression of genes involved in long stationary phase adaptation and stress-responses. Therefore, 6S RNA acts as an important regulator of transcription during the transition from exponential to stationary phase (148). Although 6S RNA-dependent phenotypes have not been fully demonstrated, it has been shown that it contributes to competitive survival and high pH resistance during stationary phase, as well as sporulation (149–151). Finally, GlmY RNA indirectly upregulates *glmS*, which encodes the enzyme GlmS that initiates bacterial cell envelope biosynthesis. Briefly, the RNA-binding protein RapZ promotes cleavage of GlmZ, another sRNA that stimulates *glmS* translation by base-pairing. However, when GlmY (that has a similar sequence to GlmZ) is expressed, it sequesters RapZ, inhibiting GlmZ decay and overall promoting *glmS* translation (152,153) (Fig. 14).

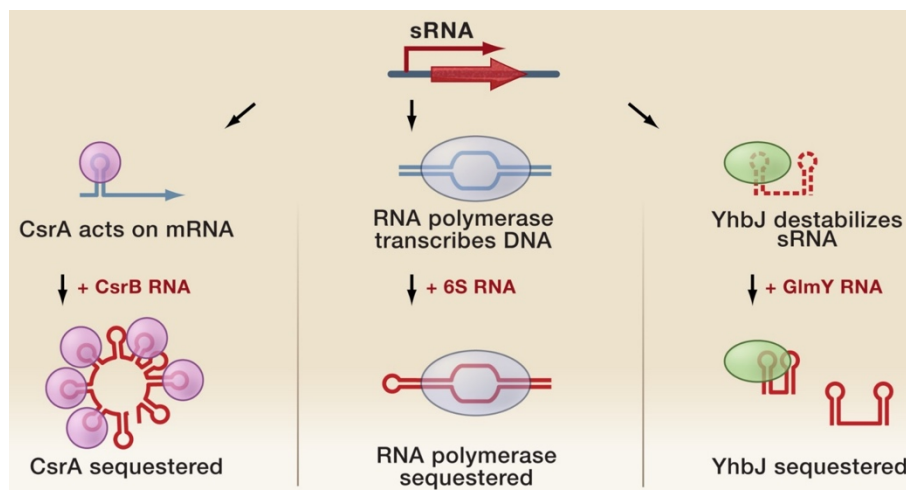


Figure 14. Protein-binding sRNAs that antagonize regulatory proteins.

Left panel: CsrA protein binds to GGA hairpins in mRNAs and alters their expression. When CsrB sRNA (red) levels increase, it sequesters CsrA, preventing its regulatory effects. Middle panel: When levels of 6S increase, the sRNA (which structurally resembles DNA during transcription) titrates RNAP away from the promoters of certain housekeeping genes reducing their transcription. Right panel: When GlmY (shorter sRNA) levels are low, YhbJ (green oval) promotes cleavage of GlmZ (longer sRNA). When GlmY is abundant, it titrates YhbJ, avoiding GlmZ degradation. Modified from (136).

In contrast to the few known examples of protein-binding sRNAs, most of the characterized sRNAs exert their regulatory functions through base-pairing to mRNAs. After binding to its mRNA target, sRNAs can promote numerous “positive” or “negative” effects, for example, i) degradation of mRNA after RNase recruitment, ii) stabilization of mRNAs after inhibition of RNase attack, iii) inhibition of translation after blocking ribosome binding, and iv) favoring mRNA translation after exposure of RBS. Although there exist other mechanisms of action, these are the most common (136,140,141) (Fig. 15).

Furthermore, depending on the location of the sRNA gene in respect of their target mRNA gene locus, sRNAs can be considered either *cis*- or *trans*-encoded.

### 2.2.1. *Cis*-encoded sRNAs

The sRNAs that are encoded in *cis* on the opposite DNA strand from the target mRNA, share extended regions of complementarity with their target, so they are often referred to as antisense RNAs (asRNAs) (154) (Fig. 15). While both transcripts are encoded in the same region of DNA, they are transcribed in opposite strands, and they interact in *trans* as diffusible molecules. However, alternative regulatory mechanisms may exist. For example, in *Clostridium acetobutylicum* the metabolism converting methionine to cysteine is controlled in *cis* via a transcriptional interference due to the collision of two divergently elongating RNA polymerase complexes, one being driven by an asRNA (155).

The majority of known *cis*-encoded antisense sRNAs are expressed from prophages, plasmids, transposons, or other mobile genetic elements. The main functions of these regulators is to maintain the copy number of the mobile elements, or to act as antitoxins that repress the translation of toxic proteins that kill the cells that have lost the mobile element (156,157).

Additionally, *cis*-encoded antisense sRNAs within the bacterial genome can modulate the expression of genes in an operon. Due to the long complementarity with their mRNA targets, when these sRNAs are in intergenic regions, they can influence both, upstream and downstream genes if they are in close proximity (Fig. 15).

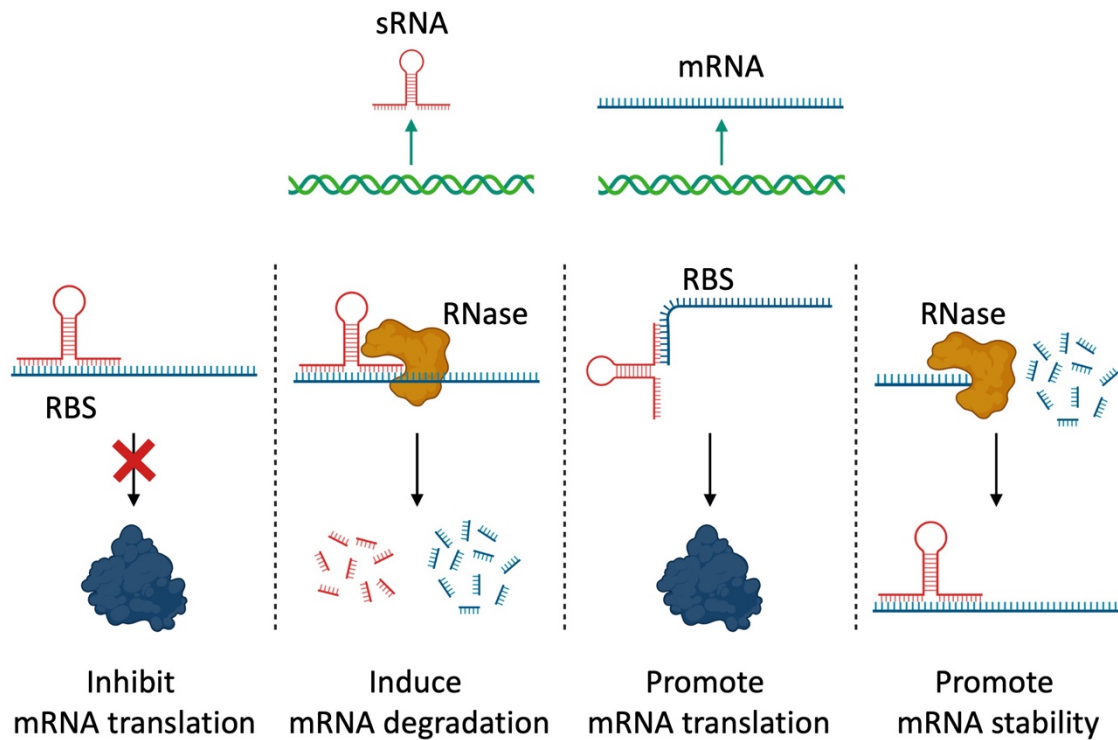


Figure 15. Gene arrangement and regulatory functions of sRNAs.

A) Two possible configurations of *cis*-encoded antisense sRNAs (red) and their target mRNA (blue) which share extensive complementarity. Left panel: sRNA encoded opposite to the 5'UTR of its target mRNA. Base pairing often blocks ribosome binding and promote target degradation. Right panels: sRNA encoded opposite to an intergenic region. Base pairing to the target mRNA usually induces RNase cleavage or transcription termination with different regulatory effects on downstream genes. B) Genes of *trans*-encoded antisense sRNAs are in a different location from the genes encoding their mRNA targets and have limited complementarity. Left panel: sRNAs can bind to the mRNA RBS and inhibit translation. Middle panel: sRNA binding to its mRNA target can induce its degradation. Right panel: sRNA binding can prevent the formation of an inhibitory structure blocking the mRNA RBS and therefore make it accessible for translation. Taken from (136).

### 2.2.2. *Trans*-encoded sRNAs

sRNAs encoded in *trans* have their loci in a distant site from their target mRNA and share only limited and short complementarity with their targets. Most *trans*-encoded sRNAs downregulate their mRNA targets by inhibiting translation after binding to the RBS, and in most cases, promoting degradation of sRNA-mRNA duplex through RNase cleavage. RNA duplex degradation is thought to increase the robustness of repression and make the regulation irreversible (158,159). However, in some cases *trans*-encoded sRNAs can exert a positive regulation after stabilizing their mRNA targets or promoting its translation after inducing conformational changes that disrupt an inhibitory secondary structure that blocks the RBS (160,161) (Fig. 15).



Most of *trans*-encoded sRNAs are expressed under highly specific growth conditions. For example, in *E. coli*, OxyS is induced by OxyR in response to oxidative stress; MicA and RybB transcription is activated by  $\sigma^F$  in response to outer membrane stress; GcvB is induced by GcvA under high glycine conditions; Spot 42 repression by CRP-cAMP is abolished at high glucose concentrations; SgrS is activated by SgrR under elevated glucose-phosphate levels; among many others (136,137,162). In fact, it is possible to consider that every major transcriptional factor could control the expression of one or multiple sRNAs.

Additionally, *trans*-encoded sRNAs interact often with multiple mRNA targets due to the possible base pairing of diverse regions of their sequence, acting as broad regulators modulating different steps of a particular physiological process, or even several more “distant” physiological processes at the post-transcriptional level. Rather than long stretches of sequence complementarity (as *cis*-encoded sRNAs), the base pairing region of *trans*-encoded sRNAs with their mRNA targets usually encompasses  $\approx 10$  to 25 nucleotides, however, in many cases it has been found that only a core of the nucleotides is critical for regulation (140,163). For example, it has been demonstrated that among the Firmicutes, RsaE downregulates mRNA targets involved in TCA cycle and arginine catabolism, through two exposed and single-stranded C-rich regions that bind to the mRNA RBS (164) (Fig. 16).

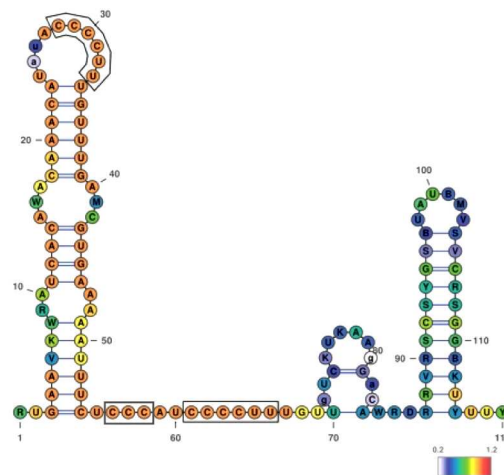


Figure 16. Predicted secondary structure of RsaE. Two exposed C-rich regions (black boxes) are responsible to base-pair with the G-rich ribosome binding site of mRNA targets and alter their translation. Modified from (164).

Since the interactions between sRNA and their target mRNA consist of a short and sometimes weak base pairing, in many cases a chaperone protein is required to mediate the interaction between the two molecules and presumably facilitate binding (165). The best-known example



of an RNA chaperone protein is Hfq. However, it has been recently demonstrated that the proteins ProQ and CsrA have also an important role for some sRNA-mRNA interactions in both Gram-positive and Gram-negative bacteria (166,167). Moreover, it has been observed that in *E. coli* a significant fraction of the ProQ-bound RNA pairs are also found associated with Hfq, indicating overlapping, complementary, or competing roles for the two proteins (168).

### 2.3. Hfq protein

Hfq is a RNA chaperone protein that acts as a homohexameric ring with two RNA binding faces. It is structurally related to eukaryotic Sm/Lm proteins, sharing a Sm-motif involved in RNA binding. Hfq facilitates regulation of *trans*-encoded sRNAs by stabilizing them, promoting structural changes of one or both RNA partners, enhancing sRNA-mRNA annealing rates and promoting RNA-duplex degradation by RNase recruitment (169–171) (Fig. 17). The importance of Hfq chaperone has been demonstrated in several *hfq* mutant strains where *trans*-encoded sRNAs are no longer active and in consequence present severe pleiotropic phenotypes with abolished virulence, impaired stress response, growth defects, among many others, reflecting its important role in sRNA-mediated regulation (172–174).

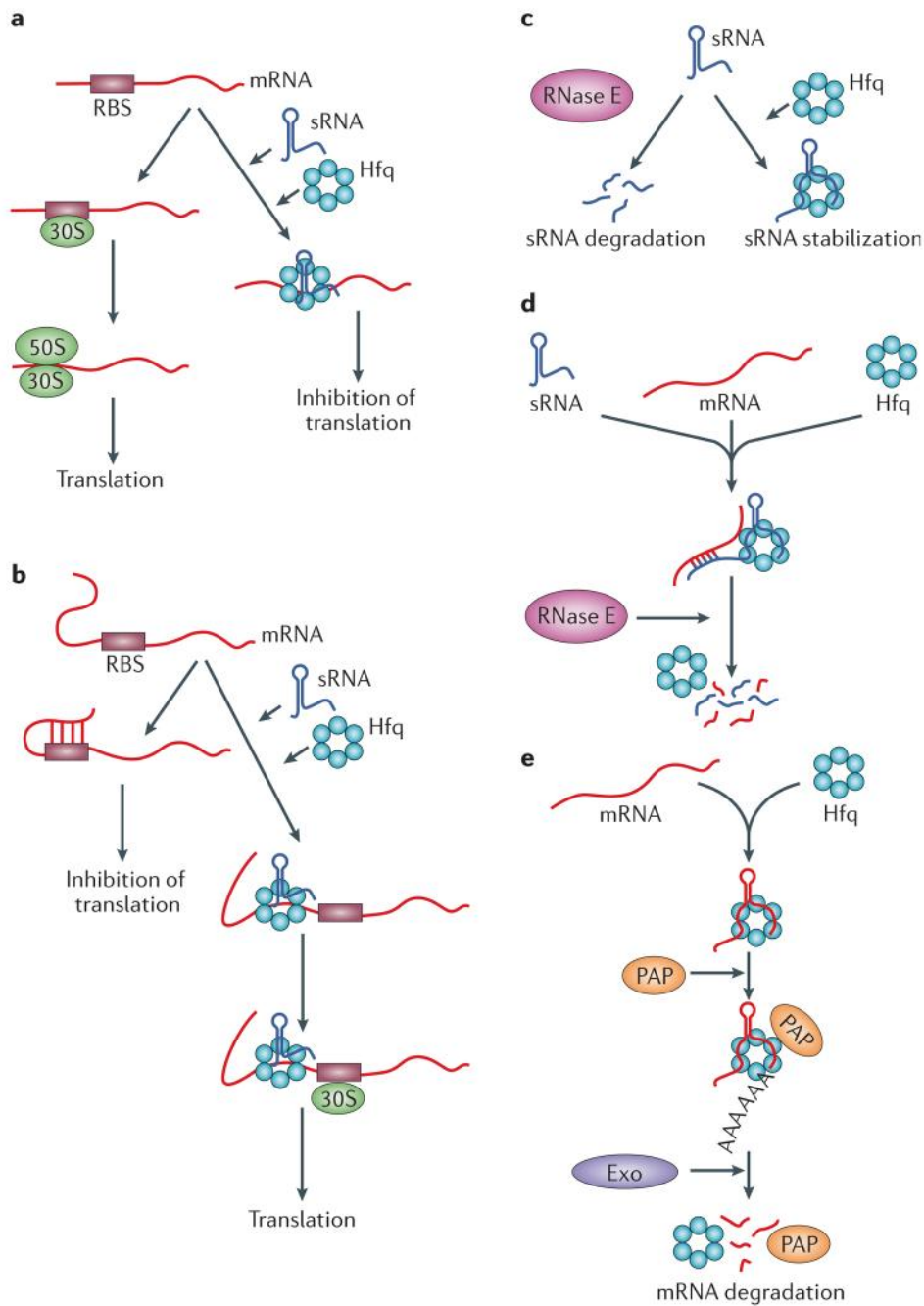


Figure 17. Models of Hfq activity.

A) Hfq in association with a sRNA might block the entry of the ribosome and repress mRNA translation. B) The secondary structure of a mRNA can mask its RBS inhibiting translation. The complex of Hfq and a specific sRNA can expose this region and facilitate translation. C) After binding, Hfq can stabilize some sRNAs and protect them from RNase-dependent degradation. D) When Hfq facilitates sRNA binding to its target mRNA, it can induce degradation of the RNA-RNA complex. E) Hfq may stimulate the polyadenylation of an mRNA by poly(A) polymerase (PAP), which in turn triggers 3'-to-5' degradation by an exoribonuclease (Exo). Taken from (169).

Despite its importance among Gram-negative species, the role of Hfq in Gram-positive bacteria remains unclear. It has been observed that many bacteria encode Hfq but do not require it for sRNA-mediated regulation, this applies to *S. aureus* (142,175–179) and quite probably to *B.*

*subtilis* (180,181). Some theories about the dispensability of Hfq among Gram-positive bacteria state that this protein may have different biochemical properties or that longer and more stable sRNA-mRNA pairings may not need Hfq intervention (182). Low GC-content has been proposed as another feature for Hfq dispensability, however, this might not be the reason since Hfq plays an essential role in *Clostridium difficile* which is 29% GC (183) and it is absent in lactobacilli with 50% GC (184). Additionally, it is likely that for specific sRNA-mRNA interactions, dedicated RNA chaperones are involved; for example, three small proteins, FbpA to C, assist the FsrA sRNA regulation of *B. subtilis* iron metabolism (185,186).

A very relevant example of a Hfq-dependent trans-acting sRNA is RyhB in *E. coli*, which is an important global regulator expressed in low-iron conditions and regulates many different cellular processes, through the so-called iron-sparing response.

## 2.4. RyhB

RyhB is the best characterized regulatory RNA of the iron-sparing response in bacteria. In *E. coli*, it is a 90 nt long sRNA, transcriptionally repressed by Fur under rich iron conditions. It was identified in 2001 by two independent genome-wide studies looking for *E. coli* sRNAs (187,188). Interestingly, a previous study in 2000 identified Fur-binding sites within a promoter located in the intergenic region between *yhhX* and *yhhY*, which now corresponds to the promoter region of RyhB (189).

In 2002, Massé and Gottesman demonstrated the Fur regulation upon RyhB, and additionally, it was shown that this sRNA requires the RNA-binding protein Hfq to exert its regulatory activities (Fig. 18). Interestingly, the sequence of *ryhB* and its promoter region (including the Fur-binding site), were shown to be conserved in *E. coli*, *Salmonella*, *Klebsiella* and in a lesser degree in *Yersinia pestis* and *Vibrio cholerae* (190).

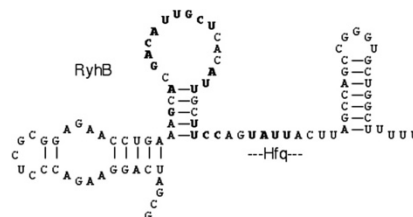


Figure 18. RyhB secondary structure. Hfq protein binds to the AU-rich exposed region of RyhB as indicated. Modified from (191).

This study was the first approach to validate RyhB targets, and resulted in six genes shown to be repressed post-transcriptionally by this sRNA under low iron conditions: *sdhCDAB* operon, encoding succinate dehydrogenase; *sodB*, encoding superoxide dismutase; *acnA*, encoding aconitase and *fumA*, encoding fumarase (both enzymes involved in the tricarboxylic acid cycle); and two ferritin genes, *ftnA* and *bfr* (190). The demonstration of downregulation of *sdhCDAB* and *fumA* by RyhB explained the growth defect observed previously in *fur* mutants using succinate or fumarate as only carbon sources. Also, it solved the paradigm of apparent “positive regulation” of Fur upon the other four mentioned genes, now considered as direct targets of RyhB (131,132). Most importantly, it was shown that upon a sudden decrease of available iron, RyhB is expressed and inhibits the production of non-essential proteins using or containing iron, helping the cell to redirect the few available iron to more vital processes. This mechanism is known as “iron-sparing response”.

An interesting claim made by Massé and Gottesman is that among bacteria, using sRNAs rather than a regulatory protein would be an economical, rapid and efficient way to have global gene repression (190). In addition to Fur inactivation in low iron conditions, RyhB contributes to iron utilization and homeostasis in bacterial cells, ultimately promoting their adaptation and survival.

#### 2.4.1. Mechanism of RyhB action

The first studies describing the molecular mechanism of RyhB activity focused mainly on *sodB* regulation encoding a superoxide dismutase, an important enzyme that protects the cell from harmful ROS (190,192). Complementarity between RyhB and *sodB* transcripts along with the activity of the RNA chaperone Hfq promote the direct interaction between the two RNA molecules. RyhB binds to a region of *sodB* mRNA containing its start codon. This interaction inhibits ribosome loading and subsequent translation of *sodB*. Upon this specific interaction, the RNA duplex is quickly degraded by the RNase E endonuclease. (Fig. 19). *E. coli* has three superoxide dismutases and SodB is the only one requiring iron. Consequently, the role of RyhB is to downregulate *sodB* for the benefit of the two other enzymes and sparing the few available iron molecules.

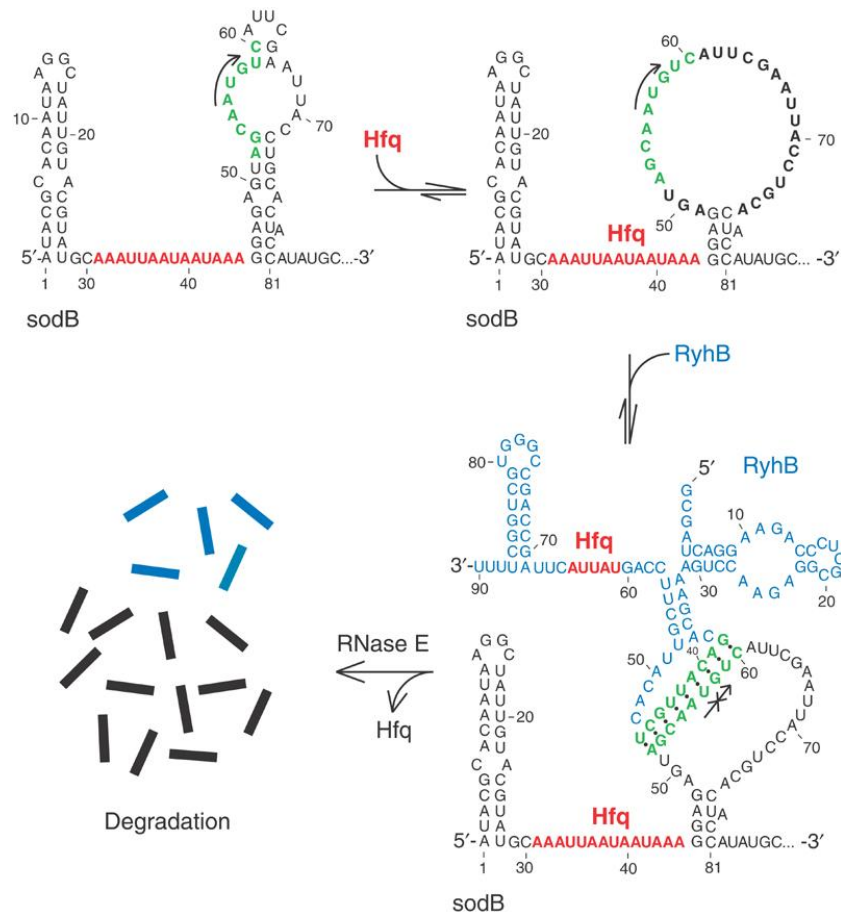


Figure 19. Model for *sodB*–Hfq–RyhB interaction.

Hfq binds to *sodB* mRNA through an AU-rich region (in red) preceding a stem-loop. This binding produces a conformational change in the stem-loop exposing the complementary region to RyhB (in green), which contains *sodB* start codon (arrow). Binding of RyhB (facilitated by Hfq), induces further conformational changes and makes the RBS at the beginning of the stem-loop inaccessible, inhibiting *sodB* translation and inducing degradation of the RNA complex by RsaE. Taken from (193).

Although mRNA degradation is usually considered to occur simultaneously with translational repression, RNase E action would contribute to gene silencing by eliminating translationally inactive *sodB*, making gene silencing irreversible. In addition to these findings, it has been shown that even when RyhB binds to *sodB* RBS, it induces RNase E cleavage at a distal site around 350 nt downstream from the RBS, within *sodB* ORF (194).

Most of the RyhB targets are regulated following the mechanism described for *sodB* (i.e., translation inhibition after direct base-pairing to target mRNA followed by RNase-mediated degradation); nevertheless, it has been shown that RyhB is able to exert its regulatory functions through a variety of different mechanisms, some of them explained with the following examples.

- Indirect regulation of *fur* translation. It has been shown that RyhB has an important role in the downregulation of its own repressor *fur*. Under rich-iron conditions, *fur* is weakly auto-regulated in order to maintain basal levels of Fur within *E. coli* (195), but under low-iron conditions, RyhB repression is alleviated and it binds to the small transcript from the *uof* gene, which is located immediately upstream *fur* and co-transcribed together with it. Subsequently, RyhB negatively affects the stability of this polycistronic transcript decreasing Fur amounts (196). This overall regulation ensures a relatively constant level of Fur for growth under high and low-iron conditions and represents the first example of indirect translational regulation by a trans-acting bacterial sRNA.

- Activation of *shiA* expression. RyhB promotes the expression of *shiA*, a gene encoding a permease for the uptake of shikimate, a precursor for siderophore synthesis in *E. coli* (197), by binding to its corresponding 5'UTR. Thus, RyhB prevents the formation of an inhibitory structure blocking the RBS within the *shiA* transcript, therefore enabling its translation (198). This regulation was the first example of a RyhB activation of a target gene expression.

- Differential regulation of *iscRSUA* transcript. RyhB controls the expression of the polycistronic *iscRSUA* transcript, which encodes a machinery for the Fe-S cluster synthesis. Under low-iron conditions, RyhB binds to a sequence overlapping the *iscS* RBS and thereafter, promotes the degradation of the region of the transcript *iscSUA* encoding precursors for the synthesis of Fe-S clusters. In contrast, the region of the transcript *iscR*, which encodes a Fe-S responsive transcriptional regulator, is exempt of RyhB-mediated degradation due to the formation of a strong secondary structure. Overall, this regulation prevents Fe-S cluster synthesis when iron is scarce, while maintaining the production of a transcriptional regulator necessary for survival under low-iron conditions (192,199). Interestingly, these were the first studies showing a differential degradation of a polycistronic mRNA mediated by a bacterial sRNA.

RyhB has been widely studied and its regulatory activity established on different targets highlights the versatility of its mode of action. Table 3 shows verified and putative targets of RyhB in *E. coli*, as well as their products and the type of regulation upon them. Further information on RyhB targets are available (192,200–202).

Target	Product	Down/Up regulation	Fe cofactor*	References
<i>acnA</i>	Aconitase (stationary phase)	Down	Fe-S	(190,192)
<i>acnB</i>	Aconitase (exponential phase)	Down	Fe-S	(192)
<i>appC</i>	Cytochrome bd-II ubiquinol oxidase subunit I	Down	Heme	(200)
<i>arnB</i>	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	Down	-	(200)
<i>bfr</i>	Bacterioferritin	Down	Heme, Fe	(190,192)
<i>bioA</i>	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Up	-	(200)
<i>cueO</i>	Multicopper oxidase	Down	(Cu <sup>2+</sup> )	(192)
<i>cydAB</i>	Cytochrome d terminal oxidase	Down	Heme	(192)
<i>dppB</i>	Dipeptide transport system permease protein	Down	-	(200)
<i>fdhF</i>	Formate dehydrogenase H	Down	Fe-S	(200)
<i>fdoGHI</i>	Formate dehydrogenase O	Down	Fe-S	(192)
<i>fdx</i>	Ferredoxin	Down	Fe-S	(192)
<i>flgB</i>	Flagellar basal body rod protein	Up	-	(200)
<i>fliC</i>	Fagellin	Up	-	(200)
<i>frdABCD</i>	Fumarate reductase (anaerobiosis)	Down	Fe-S	(192)
<i>fumA</i>	Fumarate	Down	Fe-S	(190,192)
<i>fumC</i>	Fumarate hydratase	Down	-	(200)
<i>gltB</i>	Glutamate synthase large chain	Down	Fe-S	(200,201)
<i>hscAB</i>	Chaperone proteins involved in Fe-S cluster assembly	Down	-	(192)
<i>hyaA</i>	Hydrogenase-1 small chain	Down	Fe-S	(200)
<i>hyb operon</i>	Anaerobic hydrogenase	Down	Fe-S	(192)
<i>hycA</i>	Formate hydrogenlyase regulatory protein	Down	Fe-S	(200)
<i>hydN</i>	Electron transport protein	Down	Fe-S	(200)
<i>iscRSUA</i>	Machinery for biosynthesis of Fe-S clusters	Differential regulation (see above)	Fe-S	(192,199,203)
<i>iscX</i>	Hypothetical protein	Down	-	(192)
<i>katG</i>	Catalase-peroxidase	Down	Heme	(200,201)
<i>mgo</i>	Malate:quinone oxidoreductase	Up	-	(200)
<i>mrp</i>	Protein involved in Fe-S cluster biogenesis	Down	Fe-S	(192)
<i>msrB</i>	Methionine sulfoxide reductase	Down	(Zn <sup>2+</sup> )	(192,204)

<i>narG</i>	Nitrate reductase $\alpha$ -chain	Down	Fe-S	(200,201)
<i>narK</i>	Nitrate/nitrite transporter	Down	-	(200)
<i>nirB</i>	Nitrite reductase large subunit	Down	Fe-S	(200,205)
<i>nrfA</i>	Cytochrome c-552	Down	Heme	(200,201)
<i>nuo</i> operon	NADH dehydrogenase I	Down	Fe-S	(192,206)
<i>oppB</i>	Oligopeptide transport system permease protein	Down	-	(192,200)
<i>pepB</i>	Peptidase B	Down	(Mn <sup>2+</sup> )	(192,200)
<i>pflA</i>	Pyruvate formate lyase-activating enzyme	Down	Fe-S	(192)
<i>ppc</i>	Phosphoenolpyruvate carboxylase	Down	-	(200)
<i>ppsR</i>	Phosphoenolpyruvate synthase regulatory protein	Up	-	(200)
<i>sdhCDAB</i>	Succinate dehydrogenase	Down	Heme	(190,192,206)
<i>shiA</i>	Shikimate transporter	Up	-	(192,198)
<i>sodB</i>	Superoxide dismutase	Down	Fe <sup>2+</sup>	(190,192,193,207)
<i>sseB</i>	Protein involved in serine sensitivity	Down	-	(192)
<i>tsx</i>	Nucleoside-specific channel-forming protein	Down	-	(200)
<i>ybaS</i>	Glutaminase	Down	-	(200)
<i>ybiV</i>	Sugar phosphatase	Up	(Mg <sup>2+</sup> , Mn <sup>2+</sup> , Co <sup>2+</sup> , Zn <sup>2+</sup> )	(200)
<i>ydbK</i>	Putative Fe-S oxidoreductase	Down	Fe-S	(192)
<i>ydhD</i>	Putative glutaredoxin	Down	Fe-S	(192)
<i>ydiJ</i>	Putative FAD-linked oxidoreductase	Down	Fe-S	(200)
<i>yeaC</i>	Hypothetical protein	Down	-	(192)
<i>yggG</i>	Unknown function	Down	(Zn <sup>2+</sup> )	(192)
<i>ygiQ</i>	Radical SAM superfamily protein	Down	Fe-S	(200,201)
<i>yjiM</i>	Putative dehydratase subunit	Down	Fe-S	(200)
<i>ynfEF</i>	Dimethyl sulfoxide reductase	Down	Fe-S	(200,201)

Table 3. Examples of putative and confirmed RyhB targets.

\*According to *E. coli* protein database UniProt (<https://www.uniprot.org/>).

Most of RyhB targets are involved in iron homeostasis or iron-containing proteins associated with different cellular processes. RyhB regulon is, so far, the widest controlled by a sRNA, and consequently, RyhB appears to be a master regulator of gene expression.



## 2.4.2. Other iron-responsive bacterial sRNAs

Through evolution, many bacterial species have developed a sRNA-mediated iron-sparing response to ensure their survival and proliferation when iron is scarce. Several examples of iron-responsive bacterial sRNAs among different genera, often studied in pathogenic species, are either RyhB orthologs or functional analogs.

RyhB orthologs present high sequence identities to *E. coli* RyhB, their expression is regulated by iron availability through Fur and their regulatory effects are mediated by the chaperone protein Hfq. Interestingly, some bacteria like *Salmonella enterica* or *Yersinia pestis* can harbor RyhB homologs with duplicate copies in the genome (RyhB paralogs) (190,208). Table 4 presents examples of RyhB orthologs discovered in different pathogenic bacteria, along with selected regulatory examples.

Bacterial organism	Name	Examples of regulatory functions	References
<i>Shigella flexneri</i>	RyhB	Downregulation of expression of <i>ydeP</i> , gene encoding a putative oxidoreductase required for survival under extreme acid conditions.	(209)
<i>Shigella dysenteriae</i>	RyhB	Downregulation of expression of <i>virB</i> , gene encoding a transcriptional activator of virulence-associated genes.	(210–212)
<i>Vibrio cholerae</i>	RyhB	Downregulation of expression of <i>trpA</i> , gene encoding the alpha subunit of tryptophan synthase. RyhB mutant has a defect in biofilm formation and chemotactic mobility.	(213,214)
<i>Salmonella enterica</i>	RyhB-1, RyhB-2	Downregulation of expression of <i>cyoAB</i> , genes encoding cytochrome oxidases and upregulation of <i>nirBD</i> , genes encoding the nitrate reductase. RyhB-2 mutant has an increased motility phenotype and is mainly regulated by $\sigma^5$ .	(215–217)
<i>Yersinia pestis</i>	RyhB1, RyhB2	Both RyhBs are induced in infected mice lungs, likely having a role in iron acquisition during the evolution of pneumonic plague.	(218)
<i>Klebsiella pneumoniae</i>	RyhB	Upregulation of expression of <i>orf1</i> and <i>orf16</i> gene clusters involved in capsular polysaccharide biosynthesis.	(219)

Table 4. Examples of RyhB homologs in pathogenic bacteria

On the other hand, pathogenic bacteria distant from *E. coli* possess iron-responsive sRNAs which perform an iron-sparing response comparable to RyhB. Although they are regulated by Fur and share a similar set of targets, these sRNAs do not share sequence homology and their action-

dependency upon a chaperone protein is variable. For these reasons, they are considered as RyhB functional analogs and some examples are described in Table 5.

Bacterial organism	Name	Examples of regulatory functions	References
<i>Pseudomonas aeruginosa</i>	PrrF1, PrrF2	PrrF1 and PrrF2 downregulate PA4880, a gene encoding a putative bacterioferritin and <i>antABC</i> encoding a precursor of the <i>Pseudomonas</i> quinolone signal (PQS), involved in quorum sensing.	(220–222)
<i>Neisseria meningitidis</i>	NrrF	Downregulation of <i>sdhCDAB</i> encoding for succinate dehydrogenase.	(223,224)
<i>Bacillus subtilis</i>	FsrA	Downregulation of <i>gltAB</i> , encoding the glutamate synthase and <i>citB</i> , a gene encoding the aconitase. Of note, <i>B. subtilis</i> uses FbpA, B and C as chaperone proteins for regulation of specific targets.	(185,225)

Table 5. Examples of RyhB functional analogs in pathogenic bacteria.

The aforementioned examples of iron-responsive sRNAs demonstrate their importance in a wide range of cellular processes in bacteria, as well as the diversity of regulatory mechanisms and mRNA targets, which are closely linked to the particular lifestyle of each one of these organisms.

With the emergence of new technologies and research strategies, the discovery and description of novel iron-responsive sRNA continues to expand in different bacteria. However, up to date, no sRNA fulfilling this role has been described in *Staphylococcus aureus*, a major human pathogen.

### 3. *Staphylococcus aureus*

*S. aureus* is a Gram-positive, round-shaped bacterium, member of the phylum *Firmicutes*. It is a facultative anaerobe, non-motile and does not form spores (226). *S. aureus* is an opportunistic human and animal pathogen (227–229) and a remarkably adaptable microorganism that possesses a wide repertoire of mechanisms that allow it to survive and thrive in several different environments (230).

Most commonly, *S. aureus* strains colonize asymptotically the nares, skin (*e.g.*, axillae and groin), or mucous membranes of its host. It is estimated that *S. aureus* colonizes asymptotically around 30% of the human population, while as many as 60% is intermittently colonized (227,231,232). Nevertheless, this organism can surpass the primary defense

mechanisms of the host and become an important pathogen with an accentuated severity for immunocompromised individuals, making of *S. aureus* a greatly serious community and nosocomial cause of disease (233,234). Infections by *S. aureus* can lead to a wide spectrum of morbidities, ranging from non-severe skin and soft-tissue infections, to life-threatening conditions such as bacteremia, endocarditis, necrotizing pneumonia and osteomyelitis (235–240).

Additionally, in the recent years this problem has worsen due to the increasing spread of antibiotic-resistant strains such as methicillin and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA, respectively) (241–244). Infections due to antibiotic-resistant strains are associated with higher mortality rates and increased lengths of hospital stays. MRSA accounts for at least 25 to 50% of *S. aureus* infections in hospital settings and MRSA bacteremia has mortality rates as high as 60%, an overall 2-fold increase in mortality when compared to MSSA (methicillin-susceptible *S. aureus*) (245,246). Importantly, the World Health Organization recently included *S. aureus* in its list of antibiotic-resistant "priority pathogens", a catalogue of 12 families of bacteria that pose the greatest threat to human health. Within this list, MRSA and VRSA are considered pathogens with a "high" priority for development of new antibiotics (247).

Resistance against methicillin and vancomycin is developed after the acquisition of the mobile genetic elements *SCCmec* (staphylococcal cassette chromosome *mec*) and the *vanA* operon encoded on transposon Tn1546, respectively. However, additional genomic and phenotypic features can influence the degree of resistance or sensitivity towards these antibiotics (248,249).

Besides its increasing resistance against antibiotics, *S. aureus* is a very successful pathogen due to its impressive ability to adapt and thrive in different environments. Tightly regulated metabolism shifts allow an efficient adaptation during the different steps of infection, depending on the specific conditions encountered in a particular niche (*e.g.*, pH, nutrient availability and oxygen tension) (250–252).

As a facultative anaerobic pathogen, *S. aureus* is capable to grow and produce numerous diseases in humans under aerobic, microaerophilic, or anaerobic conditions. During host infection, *S. aureus* can encounter microaerophilic and anaerobic conditions in niches such as the intestinal lumen, bone and bone marrow, within biofilms on tissues or implanted medical devices, or intracellularly within neutrophils. Moreover, through the formation of abscesses, *S. aureus* can induce hypoxia even in tissues that have relatively higher levels of oxygen, like the kidneys (253–255).

Direct sensing of oxygen by proteins is used to regulate gene expression and alter the metabolic proteome for adaptation to low oxygen tensions. A common feature among these proteins is the presence of iron-sulfur cluster cores in their sensor domain. Oxidation of the iron-sulfur core results in a conformational change that may modify the protein and/or their DNA binding or enzymatic capacity (256–258).

In the absence of oxygen, *S. aureus* can either switch to fermentative metabolism, or use other molecules than oxygen as final electron acceptors such as nitrate and nitrite(259).

### 3.1. Nitrate metabolism in *S. aureus*

In *S. aureus*, the nitrate respiration pathway is driven mainly by two enzymes, nitrate reductase (NarGHJI), that reduces nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ); and nitrite reductase (NasDEF), that further reduces  $\text{NO}_2^-$  into ammonia ( $\text{NH}_3$ ) (Fig. 20).

In bacteria, the oxidation of formate ( $\text{HCOO}^-$ ) into  $\text{CO}_2$  can serve as an electron donor process for numerous metabolic pathways including nitrate respiration (260–262). Although little is known about this process in *S. aureus*, it has been shown that in anaerobic conditions, the formate dehydrogenase Fdh oxidizes  $\text{HCOO}^-$  into  $\text{CO}_2$ , producing NADH and donating electrons to respiratory pathways, probably including the nitrate pathway (263). Moreover, it has been shown that ammonia ( $\text{NH}_3$ ), one of the many products of nitrate metabolism, can be converted to glutamate, which is further converted into  $\alpha$ -ketoglutarate that takes part within the Krebs cycle. In *S. aureus*, conversion of  $\text{NH}_3$  into glutamate is mediated by glutamate synthase Glt (264–266) (Fig. 20).

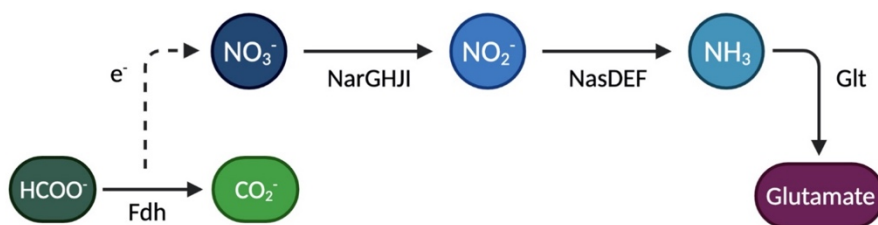


Figure 20. Nitrate metabolic pathway in *S. aureus*. Formate ( $\text{HCOO}^-$ ) conversion into  $\text{CO}_2$  by formate dehydrogenase (FdhA) might be a source of electrons for nitrate metabolism. Nitrate ( $\text{NO}_3^-$ ) is reduced to nitrite ( $\text{NO}_2^-$ ) by nitrate reductase (NarGHJI). Then,  $\text{NO}_2^-$  is reduced to ammonia ( $\text{NH}_3$ ) by the nitrite reductase (NasDEF). Finally,  $\text{NH}_3$  is converted into glutamate by the glutamate synthase (Glt).

Glutamate synthase and formate dehydrogenase enzymes are encoded in the mono- and bi-cistronic operons *gltB2* and *fdhA*, respectively; and they are expressed constitutively. However, organization and regulation of nitrate and nitrite reductases is more complex.

Nitrate and nitrite reductases contain each a iron-sulfur cluster core (267), and they are encoded in the poly-cistronic operons *narGHJI* and *nasDEF*, respectively. The transcription of both operons is regulated by the oxygen-responsive TCS NreBC in coordination with the accessory nitrate sensor NreA, encoded by the operon *nreABC*, which is located in the 3' end of the *narGHJI* operon, as a second transcript with its own promoter (Fig. 21). The *nas*, *nar* and *nre* operons are well conserved among staphylococci (268,269).

NreB is a histidine kinase that contains an O<sub>2</sub>-labile iron-sulfur cluster (270). Upon oxygen depletion, autophosphorylation of NreB activates the response regulator NreC, which binds to a GC-rich palindromic sequence (5'-TAGGGN<sub>4</sub>CCCTA-3') in the promoter region of *nasDEF*, *narGHJI-nreABC* and *narT* (which encodes a putative nitrate transporter (271)), and induces their transcription. On the other hand, apo-NreA interacts with NreB inhibiting its autophosphorylation and therefore, NreC activation. However, in the presence of nitrate, the complex NreA-NO<sub>3</sub><sup>-</sup> is formed, and the interaction with NreB is abolished. Consequently, NreB and NreC are able to induce expression of the *nasDEF* and *narGHJI* operons, as well as their own operon *nreABC* (269,272,273) (Fig. 21). By this mechanism, *S. aureus* uses nitrate and NreA to modulate the function of the oxygen sensor NreB, resulting in nitrate/oxygen co-sensing by an NreAB sensor unit as part of the NreABC system.

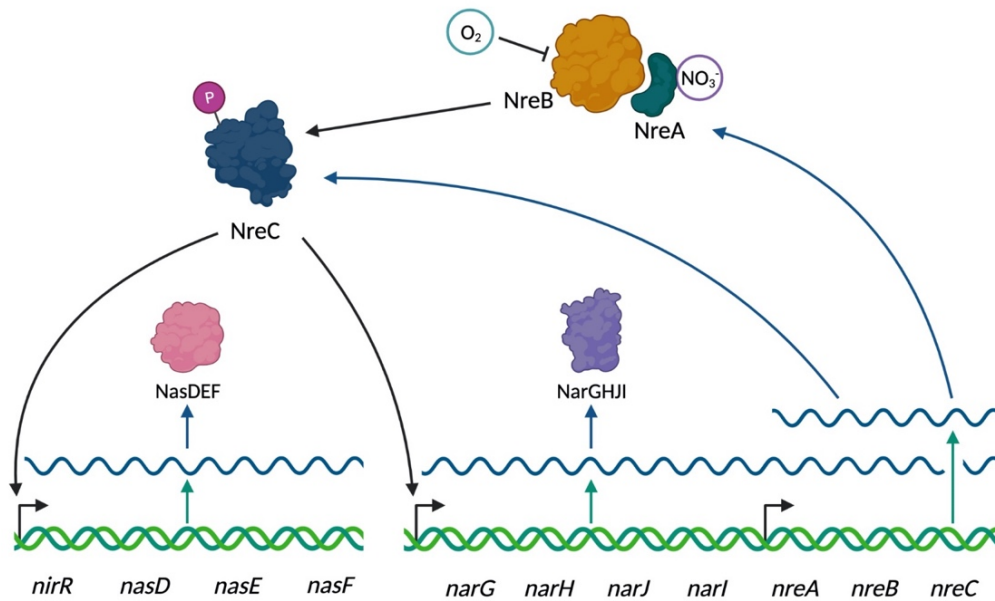


Figure 21. Regulation of operons involved in *S. aureus* nitrate metabolism.

Absence of oxygen activates NreB, in addition, the presence of  $\text{NO}_3^-$  bound to NreA alleviates its repression upon NreB, which then is able to activate NreC. Once activated, NreC promotes transcription of the operons that encode the nitrate and nitrite reductases (NarGHJI and NasDEF, respectively) as well as its own operon encoding the nitrate regulatory system NreABC. Green arrows represent transcription events, blue arrows translation events, and black arrows regulatory effects. Putative nitrate transporter NarT (also activated by NreC) is not shown since its function has not been confirmed.

In anaerobiosis, inactivation of NreABC abrogates the ability of *S. aureus* to use nitrate as the final electron acceptor, forcing the bacterium to upregulate fermentative pathways for survival (268,269).

Besides efficiently adjusting its metabolism during the different steps of infection, *S. aureus* produces numerous virulence factors that increase its pathogenesis and help it to colonize and spread within the host.

### 3.2. *S. aureus* virulence factors

Pathogenic strains from *S. aureus* produce a wide plethora of virulence factors such as toxins, cofactors for host enzyme activation, and exoenzymes, which are involved in colonization, lysis of host cells, and immune evasion or stimulation; enabling the bacterium to invade and disseminate within the host (274–276).

The main *S. aureus* toxins can be divided into three major groups: superantigens, exfoliative toxins and pore-forming toxins. The latter can be further divided into  $\alpha$ -hemolysin,  $\beta$ -hemolysin, leukotoxins and phenol-soluble modulins (PSMs) (277). In general, these toxins can damage cell membranes of the host, either by degrading inter-cellular connections or by altering immune responses, and they are related to severe diseases such as toxic shock syndrome, staphylococcal scalded skin syndrome, necrotizing pneumonia or deep skin infections (276).

Well-studied examples of pore-forming toxins are  $\alpha$ -hemolysin (Hla) and the leukotoxin Panton-Valentine Leucocidin (PVL). Hla targets a wide range of human cells including epithelial cells, endothelial cells, erythrocytes, T cells, monocytes, and macrophages, while PVL targets mainly leukocytes and neutrophils. In both cases, after being released as monomers, these toxins bind to specific proteins in the host cell membranes and recruit additional monomers. Oligomerization within the membrane results in pore formation and consequent cell damage (Fig. 22) (276,278).

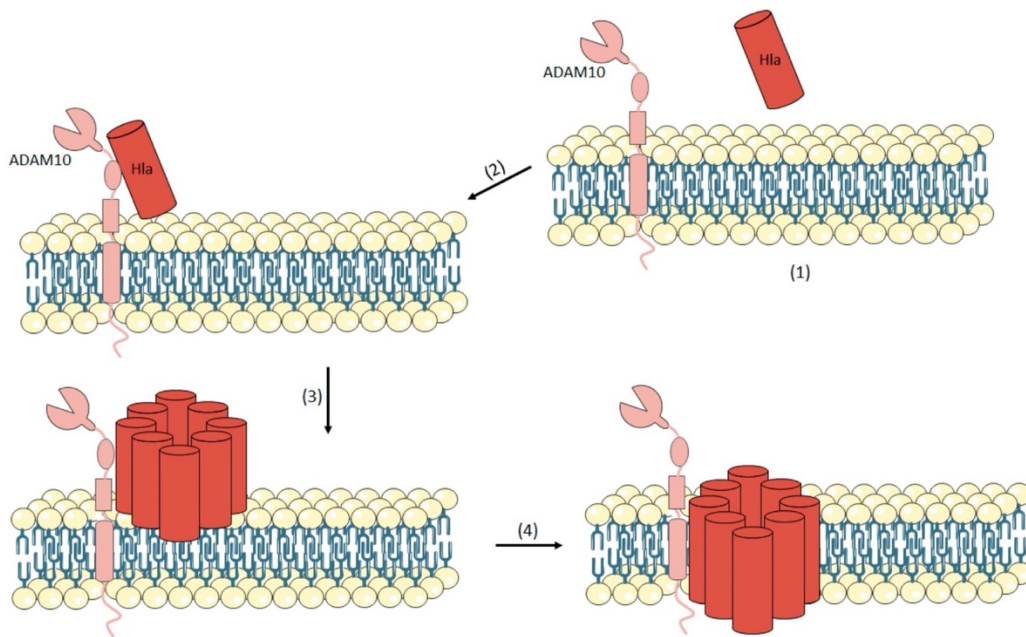


Figure 22. Mechanism of action of  $\alpha$ -hemolysin.

Hla is secreted as a monomer (1) and binds to the transmembrane protein ADAM10 (2). Then, the toxin recruits other monomers and oligomerizes into a octamer forming a pre-pore (3), to finally form a transmembrane pore (4). Taken from (276).

On the other hand, PSMs (only found in staphylococci) are lipophilic peptides that induce cytotoxicity by inserting themselves in a non-specific manner within the lipid bilayer of cell membranes leading to its disruption (275). Interestingly, it has been shown that the sRNA Teg41

regulates expression of  $\alpha$ -type PSMs, while the  $\delta$ -toxin PSM is encoded by the sRNA RNAIII (143,279).

Superantigens represent the largest family of toxins produced by *S. aureus* and include the toxic shock syndrome toxin TSST, and several staphylococcal enterotoxins. In general, these are highly stable toxins, resistant to heat, proteolysis and desiccation. Superantigens bind simultaneously to T-cell receptors and conserved structures on major histocompatibility complex class II. This reaction strongly induces the production pro-inflammatory cytokines by T-cells, causing severe systemic responses that can lead to multiple organ failure. These events affect T-cell function and prevent the generation of functional adaptive immunity (280).

Finally, exfoliative toxins are highly specific serine proteases that cause the staphylococcal scalded skin syndrome, affecting mainly neonates. These proteases induce cleavage of keratinocytes junctions and alter cell-cell adhesion in the epidermis of the host, leading to severe damages in the skin and promoting infection of deeper tissues (275,276).

In addition to the toxins, *S. aureus* also produces virulence factors that have enzymatic properties: cofactors for host enzyme activation and exoenzymes (Fig. 23). Examples of the former are coagulase, von Willebrand factor binding protein and staphylokinase. These cofactors have no enzymatic activities by themselves, but they can activate host zymogens (inactive precursors of an enzyme) involved in the coagulation cascade. By inducing coagulation within an abscess, *S. aureus* generates a protective barrier against host immune cells and overall promotes bacterial survival and dissemination. On the other hand, exoenzymes are secreted enzymes that degrade host cell components. The best examples are nucleases, proteases, hyaluronidases and lipases, which damage host nucleic acids, peptides, hyaluronic acid and lipids, respectively (275).



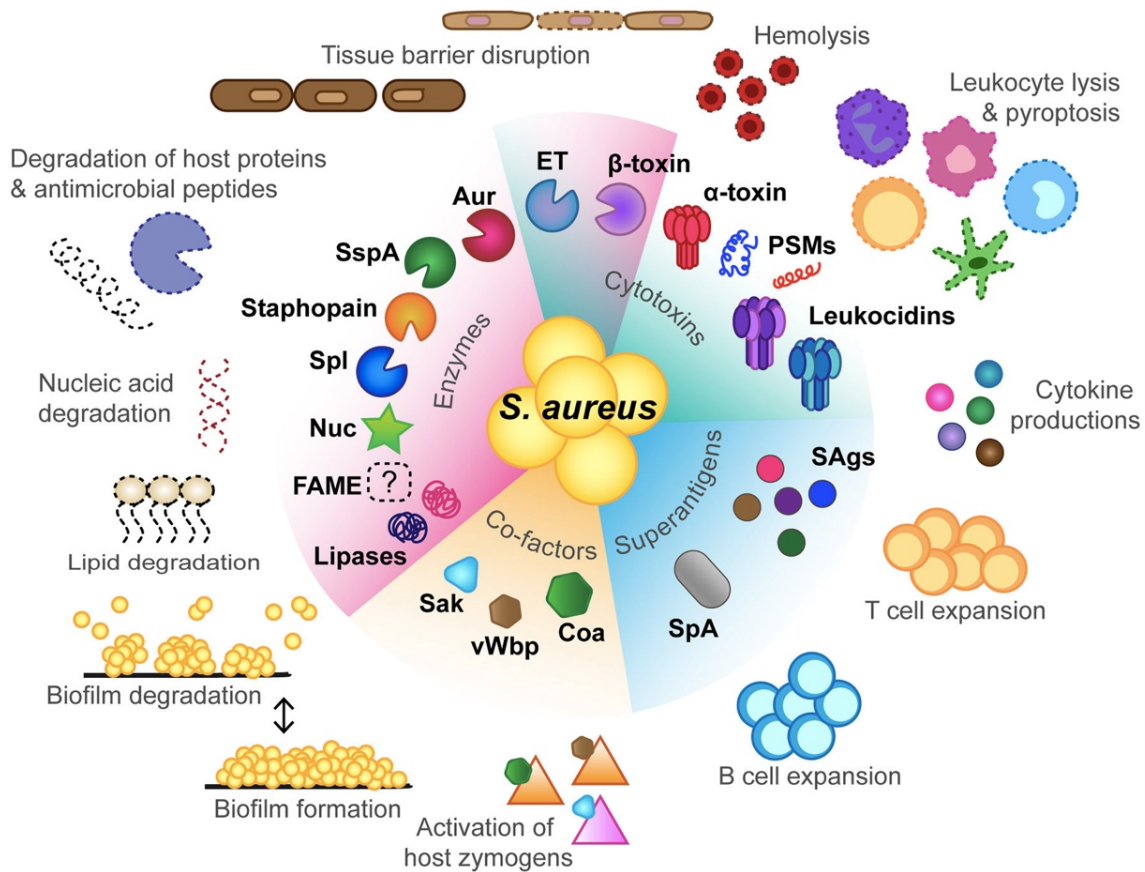


Figure 23. *S. aureus* virulence factors.

Among the different enzymes and toxins produced by *S. aureus*, superantigens affect T and B cell functions resulting in massive cytokine production and altered immune response. Cytotoxins damage numerous types of cells through recognition of specific surface receptors. Lipophilic PSMs induce cytolysis by inserting into cell lipid bilayer. Enzymes such as lipases, nucleases or proteases degrade host lipids, nucleic acids, and proteins, respectively. Some proteases can attack self-proteins to degrade biofilms and allow bacterial dissemination. Cofactors activate host zymogens in the coagulation cascade to alter clot formation and dissolution. Taken from (275).

### 3.2.1. Regulation of *S. aureus* virulence

In order to survive and adapt to different environmental niches, *S. aureus* has evolved an intricate regulatory network to control virulence factor production in both a temporal and host location manner. The genes encoding numerous virulence factors and their regulatory machinery are known as accessory genes since most of them are not essential for planktonic growth. *S. aureus* is a bacterium with a conserved core genome (281) that can acquire additional accessory genes through horizontal gene transfer of mobile genetic elements such as prophages, plasmids and the so-called staphylococcal pathogenicity islands (282).

The complex regulation of virulence factors responds to host and environment derived signals in a coordinated manner. Two-component systems (TCS) are the most common regulatory apparatus to control gene expression in response to these signals. In *S. aureus*, AgrAC, SaeRS, SrrAB and ArlRS are the most important TCS linked to regulation of virulence factors (283,284). Moreover, this pathogen can employ transcriptional factors of the SarA protein family (e.g., SarA, Rot, MgrA, etc.) and alternative sigma factors SigB and SigH ( $\sigma^B$  and  $\sigma^H$ , respectively) as additional regulators of virulence (252).

Briefly, the *agr* (accessory gene regulator) TCS is a quorum-sensing system that responds to cell density, and it is closely related to biofilm formation (285). The *agr* system consists of two transcripts, RNAII and RNAIII, whose expression is driven by the P2 and P3 promoters, respectively. The RNAII transcript is an operon of four genes, *agrBDCA*, that encode the machinery of the quorum-sensing system, while the RNAIII transcript is the major effector and regulates the expression of most *agr*-dependent target genes (286,287).

*S. aureus* constitutively produces an autoinducing peptide (AIP) that is recognized by the histidine kinase AgrC. Once that the AIP accumulates and reaches a critical extracellular concentration, it leads to autophosphorylation of AgrC and consequent activation of the response regulator AgrA, which binds to the P2 and P3 promoters and induces expression of RNAII and RNAIII. In addition, AgrA also regulates the expression of several PSMs (288). Of note, AgrD is a precursor of AIP is proteolytically processed by AgrB (252,286)(Fig. 24).

## Quorum-sensing circuit

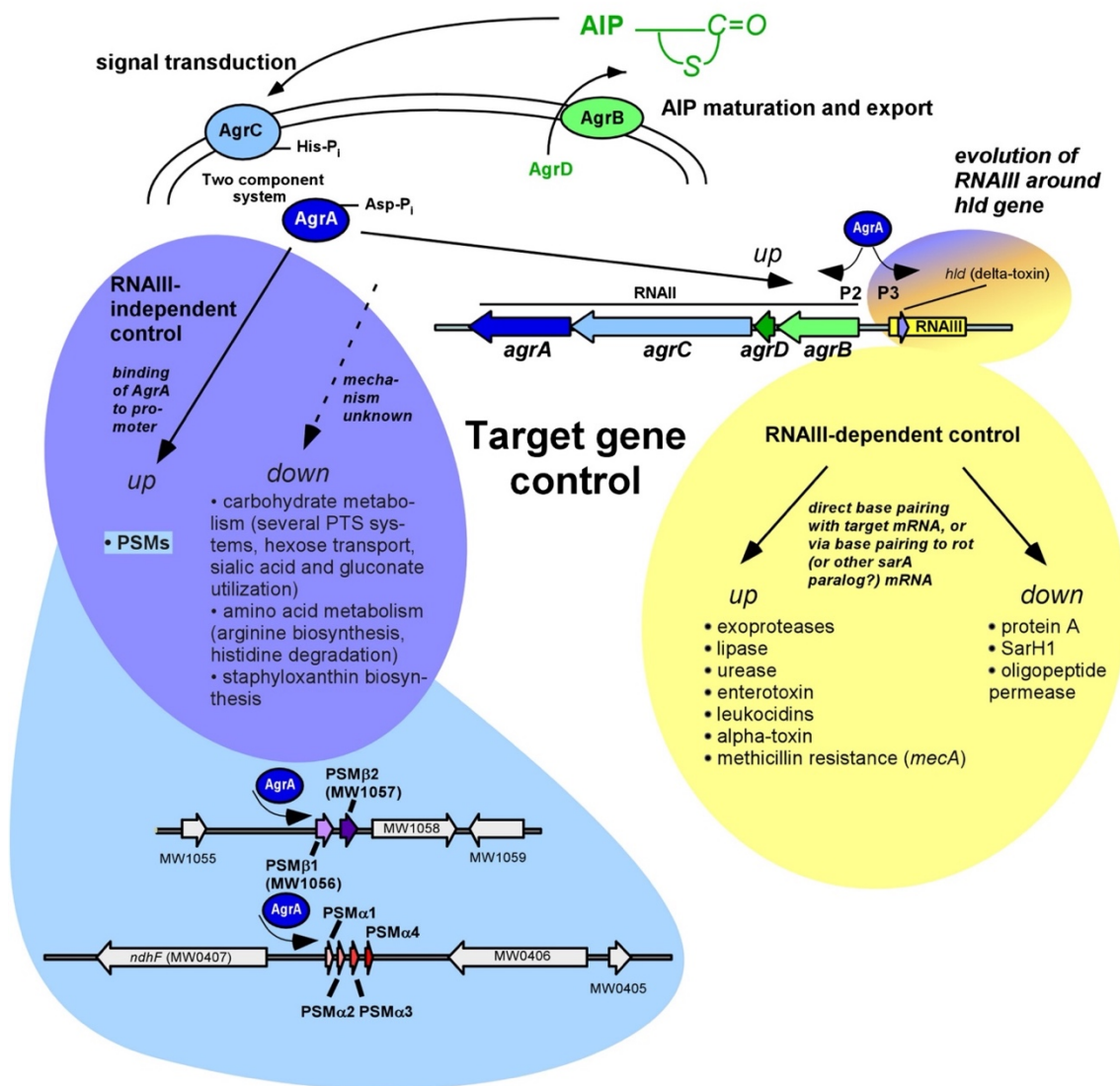


Figure 24. Quorum-sensing Agr system in *S. aureus*.

The autoinducing peptide (AIP) is produced from the AgrD precursor, modified, and exported by AgrB. When the AIP reaches a concentration threshold, it activates the AgrAC two-component system, with AgrA as the transcriptional activator of the *agrBDCA* transcript and the RNAIII transcript, which acts as the main regulatory effector of the system and encodes the  $\delta$ -toxin. While AgrA protein can also upregulate the production of PSMs, RNAIII regulates directly or indirectly (inhibition of Rot) several virulence factors including proteases, enterotoxins, leukocidins, among others. Taken from (289).

As mentioned before, RNAIII exerts its regulatory effects through mechanisms shared with many sRNAs (e.g., regulation of gene expression by base-pairing to target mRNAs). Nevertheless, this RNA with a size of 514 nt is rather long and it encodes a small protein, the  $\delta$ -toxin; these features are usually not associated with “classic” regulatory RNAs. Many well-characterized virulence factors are among the genes upregulated by RNAIII, including  $\alpha$ -hemolysin Hla, cysteine proteases (ScpA, SspB), serine proteases (SplA-F, SspA), leukocidins (LukAB, lukGH),  $\gamma$ -hemolysin Hlg, and lipase Geh. In addition, surface proteins such as protein A Spa, cell wall secretory

protein IsaA, and surface receptors (MnhA, MnhF and MnhG) are downregulated by RNAIII (143,288) (Fig. 24).

Furthermore, many virulence genes are regulated indirectly through RNAIII inhibition of the repressor of toxins Rot. During the initial stages of infection, immune evasion proteins and adhesins are upregulated while toxins and exo-enzymes are downregulated. Once the infection is established and the bacterial cell density reaches a threshold activating the Agr system, RNAIII level increases alleviating Rot-mediated repression of toxins (290,291).

Besides the AgrAC system, other important TCS involved in virulence are presented next. (for a detailed description, see (292,293)):

- The SaeRS (*S. aureus* exoprotein expression) TCS responds to host immune signals and environmental signals such as pH, salt, and glucose concentrations, or sub-inhibitory concentrations of antibiotics. It controls the production of over 20 virulence factors such as Hla, Hlb, serine protease SspA, thermonuclease Nuc, coagulase Coa, extracellular adherence protein Eap, extracellular matrix binding protein Emp, protein A, fibronectin binding protein FnbA. The transcriptional factor CodY as well as Rot and  $\sigma^B$  have been shown to negatively regulate expression of SaeRS, while Fur was reported to upregulate its expression (294–297).
- The SrrAB (staphylococcal respiratory regulator) TCS responds to low-oxygen conditions, and is one of the most important modulators of the anaerobic response in *S. aureus*. SrrAB upregulates fermentative enzymes such as lactate dehydrogenase and alcohol dehydrogenase, while it downregulates some TCA cycle enzymes such as aconitase, fumarase and NAHD dehydrogenase. Additionally, it represses transcription of virulence genes such as *agr*, *spa*, *tsst*, and *icaR* (*ica* operon repressor, see below) (252,256,259). Furthermore, SrrAB works in close relationship with NreBC TCS and Rex protein, which are responsible for nitrate metabolism and redox sensing during anaerobiosis, respectively (259,269,298).
- The ArlRS (autolysis regulated locus) TCS is a regulator of the autolytic process in *S. aureus*, which is responsible for cell division and separation through hydrolysis of different components of peptidoglycans. Interestingly, it has been found that ArlRS does not have a role in autolysis within MRSA strains. However, the precise environmental signals for ArlRS activation remain unclear (299,300). ArlRS upregulates LytRS TCS, involved in autolysis, and different extracellular

proteases that attack host antibodies; while it downregulates the global regulator MgrA, responsible to control the clumping mechanism of *S. aureus* through repression of surface proteins (e.g., Ehb, SraP, Spa and FnbB). By inhibiting MgrA, ArlRS production indirectly promotes *S. aureus* clumping and survival within host tissues (301–303). *S. aureus* clumping creates a microenvironment that enables cell growth within hosts. Progression of this bacterial growth can result in the formation of a biofilm (see below).

Among the *S. aureus* alternative sigma factors,  $\sigma^B$  responds to different stress conditions and regulates directly and indirectly around 200 genes, many of which being involved in biofilm formation, cell internalization, membrane transport and antibiotic resistance. Moreover,  $\sigma^B$  inhibits the Agr system, and therefore, it alters the expression of numerous virulence factors (304–306).  $\sigma^B$  promote bacterial aggregation by upregulating the expression of different genes encoding clumping factors and other adhesins. Additionally,  $\sigma^B$  regulates TSST through an indirect mechanism involving the virulence regulator SarA, as well as several enterotoxins (307,308). Finally, it has been shown that  $\sigma^B$  is crucial to mediate *S. aureus* adaptation during chronic infections (309,310).

### 3.3. *S. aureus* biofilms

Another mechanism that significantly increases *S. aureus* virulence and pathogenesis is its capacity to form biofilms.

Bacterial biofilms are multicellular aggregations attached to a surface, which can be host tissues or medical implants. Bacteria within biofilms are embedded in a self-produced matrix of extracellular components such as proteins, polysaccharides, and extracellular DNA; and they exhibit an altered phenotype regarding growth, gene expression and protein production. By adopting this mode of life, biofilm-embedded bacteria benefit from several advantages over their planktonic counterparts. The extracellular matrix is very resistant and protect bacteria from host immune defenses as well as external antimicrobials, while also it can concentrate the few available nutrients (311).

Although a biofilm can arise from a single cell, different environmental conditions throughout the community can induce the development of distinct subpopulations in different metabolic states (312). Some bacterial sub-populations within biofilms, although genetically identical, can

be in a so-called dormant state, where cellular division and most metabolic processes are practically halted. Since most of antibiotics target diverse processes in actively growing cells, they are not functional against these dormant subpopulations. Once that the stress conditions are removed, these dormant cells can spontaneously reverse to an active state and continue its growth and dissemination. For these reasons, biofilms are very difficult to eradicate with conventional therapies and they are an important cause for the development of chronic infections (312,313).

Biofilm development is a very complex process that is controlled by an intricate regulatory network that allows *S. aureus* to fine-tune its response to changing environmental conditions and modulate biofilm development. Since quorum-sensing enables bacteria to coordinate their behavior in a population density-dependent, one of the most important regulators of biofilm formation is the Agr system. However, many other regulators are involved in this process (285,312,313). An example of this complex regulatory network is presented next: In response to stress conditions such as nutrient deprivation,  $\sigma^B$  represses the Agr system, responsible of RNAIII expression. On the other hand, Rot inhibits the production of diverse extracellular proteases which can damage distinct components of the extracellular matrix. So, after downregulation of its repressor (RNAIII), Rot is able to prevent the production of these proteases and promote biofilm formation (314). As an alternative pathway,  $\sigma^B$  positively regulates SarA which in turn inhibits protease production (315) (Fig. 25).

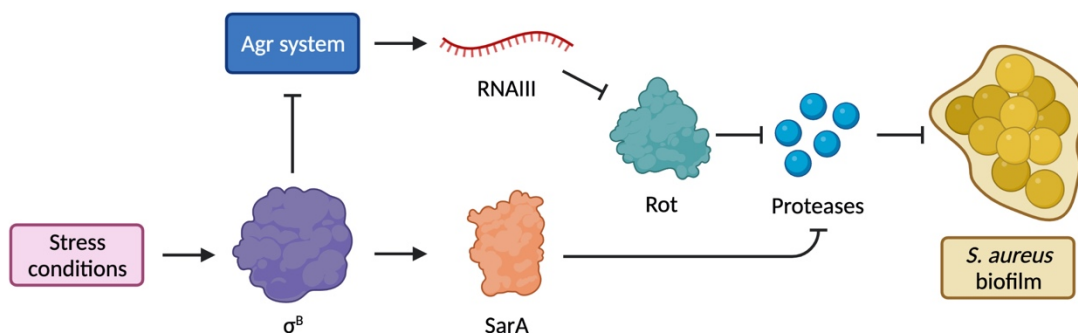


Figure 25. Example of regulatory network for *S. aureus* biofilm maintenance.

In *S. aureus*, PIA (polysaccharide intercellular antigen) is one of the main components of the biofilm extracellular matrix. PIA synthesis is mediated by the *icaADBC* operon, which in turn is upregulated by SrrAB in response to anaerobic growth, a condition often encountered within biofilms (316,317). Interestingly, it has been shown that in *S. epidermidis*, the sRNAs RsaE and

IcaZ, promote biofilm formation by upregulating PIA production after downregulation of the *icaADBC* operon repressor IcaR. In contrast to RsaE, IcaZ has not been identified yet in other staphylococci (318,319). Furthermore, *icaR* translation in *S. aureus* is influenced by its own secondary structure since a region of its long 3'UTR interacts with a complementary region of the 5'UTR that contains the RBS. This interaction hinders ribosome loading and generates a double-stranded substrate for RNase III, in overall promoting biofilm formation (320).

Numerous additional examples of regulatory mechanisms exist around biofilm formation and its maintenance (285,312,313,321); and given the importance of biofilms in *S. aureus* chronic and infections, numerous studies focus on this complex mechanism of bacterial adaptation and survival in order to counteract staphylococcal pathogenesis (322–324).

During infection, besides the rapid metabolic adjustments, the production of virulence factors, the resistance against antimicrobials and possible formation of biofilms, *S. aureus* is notably capable to obtain essential nutrients from the host, including iron.

### 3.4. Iron acquisition in *S. aureus*

Iron is essential for *S. aureus* survival, colonization, and subsequent pathogenesis during infection. As many other human pathogenic bacteria, *S. aureus* has developed complex mechanism to obtain iron from the host, circumventing nutritional immunity. Since most of the bacterial mechanisms for iron acquisition have been described (pg. 20), only specific systems of *S. aureus* will be presented next.

Most of iron acquisition systems in *S. aureus* are regulated by Fur (325). In addition, PerR (a Fur homolog), is a manganese-, iron- and peroxide-responsive transcriptional repressor that binds to a specific motif (atTataATTATTATaAt) in the promoter region of its target genes. PerR regulates the expression of iron storage proteins such as ferritin Ftn, the ferritin-like MrgA and bacterioferritin comigratory protein Bcp. It also controls the oxidative stress response by regulating the transcription of proteins such as proteins catalase KatA, alkyl hydroperoxide reductase AhpCF, and thioredoxin reductase TrxB. Interestingly, PerR regulates its own transcription, as well as *fur* transcription. The indirect control of iron homeostasis by PerR regulation of Fur and the direct control of iron storage proteins allow *S. aureus* to coordinate

the intracellular availability of free iron with the level of antioxidant proteins present in the cell, a possible adaptation mechanism for intracellular survival (114,122,326).

#### 3.4.1. Siderophore-mediated iron acquisition

*S. aureus* obtains extracellular iron complexed to host proteins transferrin and lactoferrin using two siderophores, staphyloferrin A and staphyloferrin B (327). Both siderophores are synthesized via the non-ribosomal peptide synthetase independent pathway and they are part of the carboxylate family of siderophores. Staphyloferrin A is encoded by the *sfaABCD* operon, and its transporter HtsABC, has been found to also mediate heme transport, acting as a promiscuous system involved in the transport of multiple iron sources (328–330). On the other hand, staphyloferrin B is encoded by the *sbnABCDEFGHI* operon and is imported into the cell by the SirABC transporter (331,332). The genes involved in the biosynthesis and transport of staphyloferrin A and B are regulated by Fur and therefore expressed under low-iron conditions (333,334) (Fig. 26).

Despite *S. aureus* does not generate hydroxamate-type siderophores, it produces the ferric hydroxamate uptake transporter FhuCBG, which encodes the ATPase FhuC that is necessary for function of the transporters of staphyloferrin A and B. In addition, the FhuCBG transporter, with help of the lipoprotein receptors FhuD1 and FhuD2, is able to recognize and use xenosiderophores as a source of iron. Transcription of *fhuCBG* and *fhuD1/2* is regulated by Fur (335–337) (Fig. 26). In the same manner, although *S. aureus* does not produce catechol-type siderophores, it can use them via the staphylococcal siderophore transporter Sst (334,338).





### 3.4.2. Iron acquisition from heme-iron

Heme-iron is the preferred source of iron for *S. aureus* during infection; by using hemolysins, it is able to lyse erythrocytes and release hemoglobin (334,339). Moreover, due to its thick peptidoglycan layer, *S. aureus* has developed a complex heme transport mechanism, known as the Isd system, which is encoded within five operons: *isdA*, *isdB*, *isdCDEF srtB isdG*, *isdH*, and *orfX isdI*. Fur regulates the transcription of all these operons (334,340).

IsdA, IsdB and IsdH are receptors covalently anchored to the cell wall by sortase A SrtA, while IsdC is a transporter that uses sortase B SrtB. On the other hand, IsdDEF is an ABC transporter complex that mediates transport of heme into the cytoplasm.

The current model for Isd-mediated transport proposes that IsdA, IsdB and IsdH are surface-exposed hemoprotein receptors that pass heme to IsdC, which then transports heme through the cell wall to the membrane localized IsdDEF ABC transport system (341) (Fig. 26). While IsdB receptor targets hemoglobin, IsdH targets the haptoglobin-hemoglobin complex (342–344). IsdA, IsdB, IsdH and IsdC contain NEAT domains that bind reversibly to heme molecules (345). Through these NEAT domains, heme can be transferred i) from IsdB to either IsdA or IsdC, ii) from IsdA to IsdC, iii) from IsdB or IsdC to membrane-bound IsdE, but not from IsdA, or iv) from IsdH to IsdA, IsdB, IsdC and IsdE. This network highlights the complex cooperation among the Isd elements for an efficient iron transport from different sources (341,346–348).

Compared to the understanding of iron transport across the cell wall, less is known about the details of its transport across the membrane. The current model of IsdDEF function is that the IsdE lipoprotein receives heme and then passes it to IsdF, the ABC permease, which transports heme through the membrane using energy provided by the ATP hydrolyzing activity of IsdD (341,347). Once that heme-iron enters the cytoplasm, the heme-degrading proteins IsdG and IsdI release iron so it can be used within the cell (349,350).

As mentioned before, besides the Isd system, *S. aureus* can use heme-iron through the Fur-regulated heme transport system Hts (330) (Fig. 26). Additionally, a system for heme detoxification has been proposed in *S. aureus*. The ABC-type heme regulated transporter HrtAB, is thought to function as an efflux pump that expels a toxic metabolite that accumulates because of heme exposure. While the ATPase activity of HrtA has been characterized, the permease activity of HrtB has yet to be confirmed. Moreover, the substrate of HrtAB and the mechanism

by which HrtAB detoxifies heme are unknown. The transcription of *hrtAB* is regulated by the two-component system known as the heme sensor system (HssRS), which is activated by heme over-exposure (Fig. 27). Overall, it has been demonstrated that both HrtAB and HssRS are essential for survival against heme cellular toxicity (351–354).

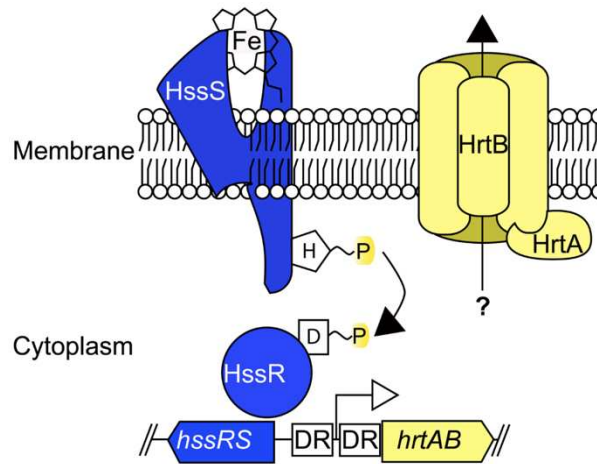


Figure 27. Sensing and alleviation of heme-associated toxicity.

HssS is autophosphorylated after heme exposure through an unknown mechanism, which results in HssR activation. HssR binds to a direct repeat sequence (DR) within the *hrtAB* promoter region resulting in the expression of HrtA ATPase and the HrtB permease, which together form an ABC-type transport system that alleviates heme-mediated toxicity through an unknown mechanism. Taken from (327).

All above-mentioned processes involved in *S. aureus* survival and dissemination are tightly regulated by elements such as TCS, transcriptional factors,  $\sigma$ -factors, and RNA-binding proteins. However, the role of sRNAs as regulators of numerous metabolic and pathogenic processes has become greatly acknowledged.

### 3.5. sRNAs in *S. aureus*

sRNAs are important regulatory elements for *S. aureus* adaptation and virulence (355–360), and several studies have focused on the identification of sRNAs via bioinformatics, next-generation sequencing (NGS), and other experimental approaches (361–376). From these studies it has been shown that regulatory RNAs in *S. aureus* are heterogeneous in size, structure, mechanism of action, and function; and they are transcribed from both, the core and accessory genome, under specific growth phases and environmental conditions. However, a shared feature among *S. aureus* sRNAs is its apparent Hfq-independence for regulation. It has been demonstrated that the deletion of *hfq* in *S. aureus* does not produce any phenotype when tested on over 1500

growth conditions (175,176). Moreover, no sRNA-chaperone proteins have been detected so far in *S. aureus*, in contrast to model bacteria such as *E. coli* (168,377–379) and *B. subtilis* (167,185,186).

Next, three selected examples of regulatory RNAs will be briefly described in order to demonstrate their versatile roles within *S. aureus*.

### 3.5.1. RsaE

RsaE, known as RoxS in *B. subtilis*, is a highly conserved sRNA among the Bacillales order, including the genera *Staphylococcus*, *Bacillus*, *Micrococcus*, *Geobacillus*, among others. Through these conservation studies, it was found that RsaE/RoxS contain three C-rich motifs, later recognized to be important for mRNA interaction involving ribosomal binding sites (164,177,319,380,381) (Fig. 16). This unusual conservation across bacterial families and genera indicates a selective pressure to maintain the sequence and structure of RsaE, and leads to a prominent function of this regulator in the physiology of low-GC Gram-positive bacteria (382).

The most common mechanism of action of RsaE consist in inhibition of transcription of its mRNA targets through direct interaction of the C-rich motifs with the mRNA 5'UTR including the RBS (G-rich), and subsequent mRNA degradation by RNase III (164,319,383). However, it has been shown that RoxS can positively regulates mRNA stability and translation of an mRNA (*yflS*) by preventing RNase J1-dependent exonucleolytic degradation (384).

Studies on RsaE/RoxS have demonstrated that this sRNA has important roles within the regulation of important metabolic pathways such as the TCA cycle, amino acid metabolism and folate-dependent one-carbon metabolism, with regulatory links existing towards control of programmed bacterial cell death and biofilm formation (164,177,319,365). For a detailed review on RsaE and its different validated direct mRNA targets and influenced cellular functions, see (381).

Except for succinate/fumarate conversion, all TCA cycle steps have been found to be negatively influenced by RsaE (Fig. 28). Within growing bacteria, the TCA cycle is usually repressed in early growth stages when sufficient glucose is available, and activated during stationary growth when glucose is depleted. The main purpose of the TCA cycle is to generate reducing power through

oxidation of substrates and the transfer of electrons to NAD<sup>+</sup> and FAD to yield NADH and FADH<sub>2</sub>, which are used to drive ATP synthesis during oxidative phosphorylation. In addition, NADH and FADH<sub>2</sub> serve as reducing agents in numerous cellular redox reactions. RsaE has been found to negatively influence several oxidoreductases, and many of these downregulated proteins use NAD and FAD as prosthetic groups to accomplish their oxido-reduction/electron transfer reactions (Fig. 28). Therefore, balancing cellular NAD<sup>+</sup>/NADH ratios in response to nutrient availability is now considered to represent the most likely primary function of RsaE in Gram-positive bacteria. This role is in good agreement with demonstrations of growth phase-dependent RsaE transcription, with pre-stationary RsaE accumulation and a decrease in stationary phase, suggesting indeed a role of RsaE in adaptation to nutrient availability and growth phase changes (164,177,365,380).

RoxS also facilitates malate uptake by stabilizing *yfiS* mRNA, encoding a malate transporter. Malate is an important carbon source for NADH/NAD<sup>+</sup> production through the TCA cycle (384).

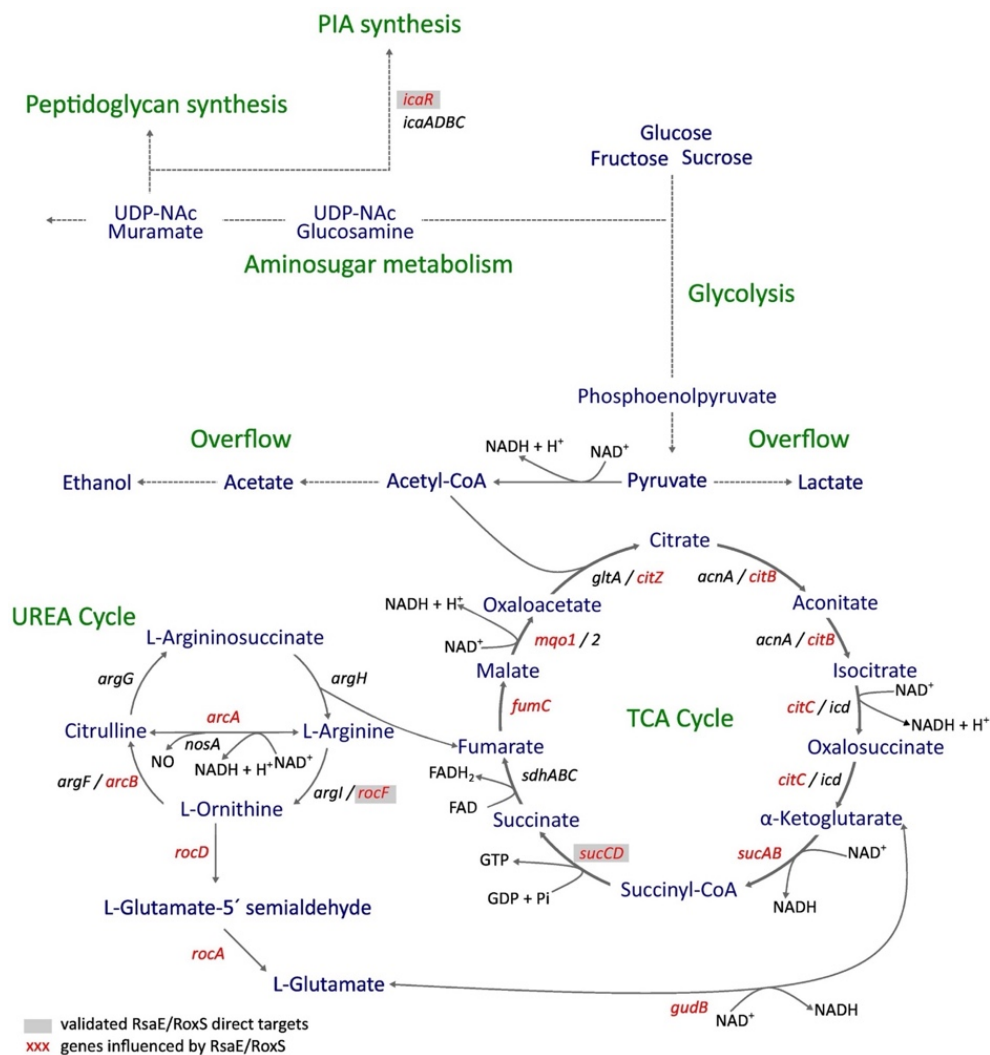


Figure 28. Overview on selected central carbon flux pathways influenced by RsaE. Verified RsaE targets are in grey boxes. Genes known to be influenced by RsaE in *S. aureus*, *S. epidermidis* and/or *B. subtilis* are in red color (381). Taken from (381), which is an adaptation of an original figure from (164).

It has been shown that in both *S. aureus* and *B. subtilis*, RsaE/RoxS transcription is activated by the TCS SrrAB/ResDE in response to increasing NO levels in the growth medium, and in consequence, RsaE downregulates several mRNA targets with functions related to oxidative stress and oxidoreduction reactions, suggesting feedback control of RsaE expression in response to the redox status of the cell. Importance of the redox equilibrium for RsaE regulation is further highlighted by its repression by the transcriptional factor Rex (380).

Finally, RsaE has been found to be expressed in populations of the model biofilm-forming bacterium *S. epidermidis*, where it contributes to biofilm development by directly targeting mRNAs involved in PIA synthesis control (*i.e.*, *icaR*), metabolic reprogramming (*i.e.*, *sucCD*) and extracellular DNA release (*i.e.*, *lrgA*) (319). Of note, *icaR* mRNA is also a putative target of RsaE in *S. aureus* (164). Additionally, downregulation of the TCA cycle by RsaE may trigger metabolic reprogramming in favor of biofilm matrix production.

### 3.5.2. RNAIII

The regulation of RNAIII expression has been already described (pg. 56). Therefore, only some additional details about its regulatory mechanisms will be presented next.

Through RNAIII, the quorum sensing Agr system controls *S. aureus* transition from a colonization mode, where the expression of several surface proteins and adhesins is upregulated, to an infection mode with the subsequent production of numerous virulence factors and inhibition of elements used for colonization (385) (Table 6, Fig. 29).

The secondary structure of RNAIII reveals that this RNA contains several C-rich sequence motifs present in apical loops and unpaired regions that were identified as seed sequences for binding with the RBS of several target mRNAs, inhibiting their translation, and in some cases, promoting RNA-duplex degradation (143,386) (Table 6, Fig. 29).

Gene	Protein	Functions	RNAIII-dependent regulation	References
<i>spa</i>	Protein A	Adhesion, immune evasion	Translation repression, RNase III degradation	(387)
<i>coa</i>	Coagulase	Adhesion, fibrin clot formation	Translation repression, RNase III degradation	(388)
<i>sbi</i>	Sbi	Adhesion, immune evasion, activates complement C3	Translation repression	(389)
<i>sa1000</i>	SA1000	Adhesion, fibrinogen-binding protein	Translation repression, RNase III degradation	(142)
<i>lytM</i>	LytM	Cell wall metabolism, protein A release	Translation repression	(390)
<i>rot</i>	Rot	Repressor of toxins	Translation repression, RNase III degradation	(142,391,392)
<i>mgrA</i>	MgrA	Repressor of cell surface proteins, activator of capsule, inhibitor of autolysis	mRNA stabilization	(393)
<i>hla</i>	Hla	Pore-forming toxin, induces apoptosis	mRNA structural changes, translation activation	(394)
<i>hld</i>	Hld	PSM toxin, hemolysis, cytolysis	Encoded by RNAIII	(386,395)

Table 6. Direct targets of *S. aureus* RNAIII. Modified from (143).

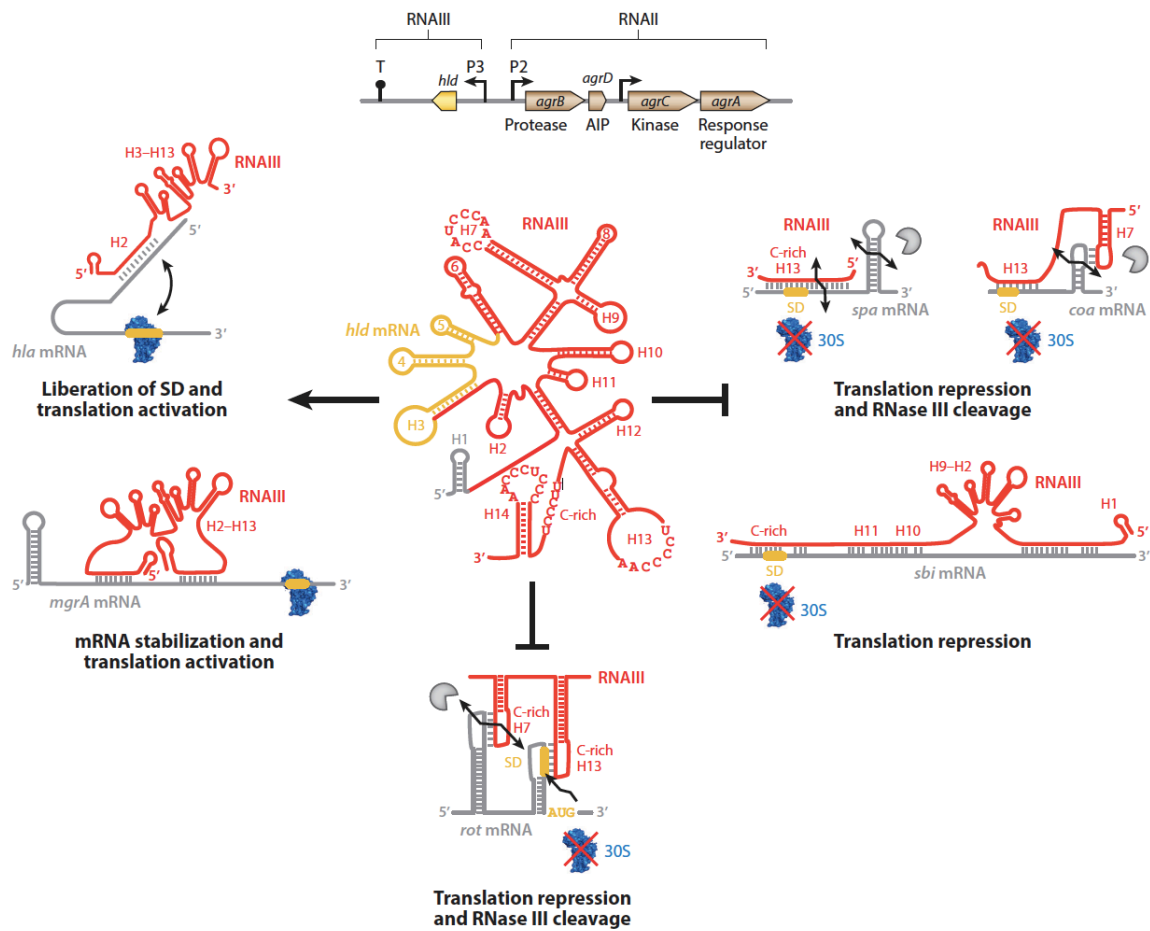


Figure 29. Mechanism of action of RNAIII on its target mRNAs.

Genomic organization of the quorum-sensing Agr system is given at top. The schematic secondary structure of RNAIII (red) is from (386). The *hld* gene encoding  $\delta$ -hemolysin is yellow. The various C-rich sequence motifs of RNAIII are the seed sequences that bind to the RBS of mRNA targets (gray). Various topologies of RNAIII-mRNA are given. Binding of RNAIII to several mRNAs (*spa*, *coa*, *rot*, *sbi*) repress their translation by preventing binding of the 30S small ribosomal subunit. In several cases this step is followed by rapid degradation initiated by the double strand-specific RNase III (gray circle). Binding of RNAIII to target mRNAs can also activate synthesis of exotoxins. For *hla* mRNA, interaction with RNAIII prevents the formation of an inhibitory structure sequestering the RBS, whereas for *mgrA* mRNA, binding of RNAIII stabilizes the mRNA against RNase degradation. Taken from (143).

The critical role of RNAIII and the Agr system in *S. aureus* virulence has been evident in Agr-defective strains that have lost their ability to disseminate in patient tissues (396,397). Therefore, in the last years, numerous efforts have been made targeting RNAIII and the Agr system in order to counteract *S. aureus* pathogenesis (285,398–404).

### 3.5.3. SprX

Since the spread of *S. aureus* strains resistance to  $\beta$ -lactams (*i.e.*, MRSA), glycopeptide antibiotics (*e.g.*, vancomycin and teicoplanin) have become the last therapeutic line of defense against



multi-resistant *S. aureus* infections (249,405–407). In *S. aureus*, methicillin and glycopeptide resistance, as well as the expression of extracellular nuclease, lipase and protease is modulated by the  $\sigma^B$ -controlled *yabJ-spoVG* operon (408,409).

Recently, it was proposed that the sRNA SprX (small pathogenicity island RNA X) (365), influences bacterial resistance to glycopeptide antibiotics by controlling SpoVG expression through binding of a C-rich region to *spoVG* RBS. This binding prevents ribosomal loading onto *spoVG*, and specifically inhibits translation of the second downstream gene within the *yabJ-spoVG* operon without altering the stability of the *yabJ-spoVG* mRNA. Absence of SprX increases *S. aureus* resistance to teicoplanin while its over-expression reduces it (410). Additionally, it has been proposed that SprX is implicated into the response towards other antimicrobials such as benzalkonium and PVP-iodine (411). These studies highlight the importance of sRNAs within the antibiotic resistance in *S. aureus*.

Additionally, SprX has been shown to impact *S. aureus* virulence by upregulating the expression of virulence genes, such as the cell wall-associated clumping factor B ClfB, the secreted Hld (encoded within RNAIII transcript), and interestingly, the autolysin regulator WalR, which in turn regulates the expression of AtIA, IsaA, and LytM autolysins (412,413) (Fig. 30).

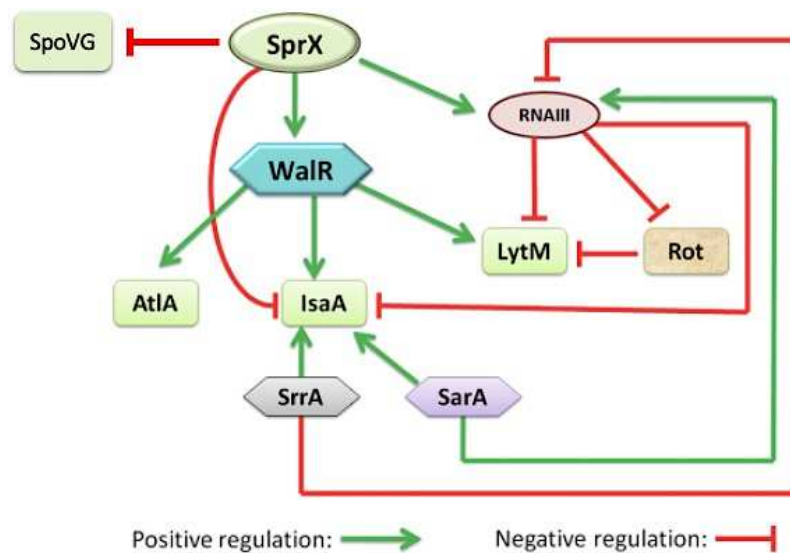


Figure 30. Regulatory network of SprX in *S. aureus*. SprX upregulates expression of WalR, which in turn regulates the expression of AtIA, IsaA, and LytM. Besides, SprX directly modulates the expression of IsaA and RNAIII. The expression of IsaA and LytM is additionally controlled by different regulators (SrrA, SarA, RNAIII, Rot). Additionally, SprX downregulates expression of SpoVG. Modified from (413).

## H. OUTLINE OF THE THESIS

Numerous sRNAs have been identified in the human pathogen *S. aureus*, and so far, their crucial role as global regulators has been demonstrated for few of them. Different mechanisms of survival and pathogenesis in *S. aureus*, such as metabolic adaptation to nutrient availability, production of virulence factors, and antibiotic resistance, are modulated by sRNAs. However, prior to this work, no sRNA involved in *S. aureus* iron homeostasis had been reported. Therefore, the objective of this thesis was to identify and characterize the potential sRNAs contributing to the regulation of iron homeostasis in *S. aureus*, a process of vital importance during infection.

# I. sRNA-CONTROLLED IRON SPARING RESPONSE IN STAPHYLOCOCCI

## sRNA-controlled iron sparing response in Staphylococci

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### ABSTRACT

*Staphylococcus aureus*, a human opportunist pathogen, adjusts its metabolism to cope with iron deprivation within the host. We investigated the potential role of small noncoding RNAs (sRNAs) in dictating this process. A single sRNA, named here IsrR, emerged from a competition assay with tagged-mutant libraries as being required during iron starvation. IsrR is iron-repressed and predicted to target mRNAs expressing iron-containing enzymes. Among them, we demonstrated that IsrR down-regulates the translation of mRNAs of enzymes that catalyze anaerobic nitrate respiration. The IsrR sequence reveals three single stranded C-rich regions (CRRs). Mutational and structural analysis indicated a differential contribution of these CRRs according to targets. We also report that IsrR is required for full lethality of *S. aureus* in a mouse septicemia model, underscoring its role as a major contributor to the iron-sparing response for bacterial survival during infection. IsrR is conserved among staphylococci, but it is not ortholog to the proteobacterial sRNA RyhB, nor to other characterized sRNAs down-regulating mRNAs of iron-containing enzymes. Remarkably, these distinct sRNAs regulate common targets, illustrating that RNA-based regulation provides optimal evolutionary solutions to improve bacterial fitness when iron is scarce.

## INTRODUCTION

Iron is essential for numerous enzymatic processes that use its redox properties, notably in respiration. Despite being one of the most abundant element on earth, iron bioavailability can be limiting. The mammalian host may prevent pathogen proliferation by restricting access to free iron, creating a nutritional immunity. In parallel, pathogens have developed complex systems for iron assimilation leading to a “battle for iron” (1-4). While iron is required for growth, its excess is toxic. Indeed, ferrous iron decomposes hydrogen peroxide via the Fenton reaction into oxygen radical species that are highly reactive and oxidize a wide range of substrates, including bacterial macromolecules (DNAs, RNAs, proteins and lipids). Since most living organisms need iron but are sensitive to its excess, a strict regulation of iron uptake and iron-containing enzymes is necessary (5,6).

For many bacteria, the main contributor to iron homeostasis is the transcriptional regulator Fur (7). In the presence of iron, Fur acts as a repressor targeting genes involved in iron acquisition, thereby ensuring a feedback regulation to maintain homeostasis of intracellular iron. In *Escherichia coli*, the absence of Fur leads to repression of a subset of genes using iron as cofactor by an indirect effect. The mystery of this apparent paradox was solved by the discovery of a small regulatory RNA (sRNA) repressed by Fur, RyhB (8). In low iron conditions, RyhB, assisted by the RNA chaperone Hfq, accumulates and pairs to mRNAs, preventing the expression of iron-containing proteins and inducing an iron-sparing response (9). RyhB orthologs are present in many species from the *Enterobacteriaceae* family where they contribute to adaptation to low iron conditions (5,10). In *Pseudomonas* species, a major contributor to this adaptation is PrrF, a sRNA down-regulated by Fur contributing to iron homeostasis (11). Of note, the corresponding *ryhB* and *prfF* genes are duplicated in some species. Surprisingly, much less is known concerning Gram-positive bacteria where, so far, RyhB and PrrF orthologs were not found. sRNAs can nevertheless be regulators of the iron-sparing response. In *Bacillus subtilis*, FsrA sRNA is down-regulated by Fur, and with the help of three RNA chaperones, FbpABC, it prevents the expression of iron-containing enzymes (12-14). In *Mycobacterium tuberculosis*, the sRNA Mrsl is predicted to be down-regulated by IdeR,

a functional homolog of Fur, and to exert a similar iron-sparing response. Interestingly, this bacterium does not possess Hfq (15).

*Staphylococcus aureus* is a human and animal opportunistic pathogen and a leading cause of nosocomial and community acquired infections (16,17). Its success as a pathogen relies on the expression of numerous virulence factors and its adaptability to various environmental conditions including metal starvation. Indeed, iron limitation in the host is counteracted by alleviation of Fur repression, leading to the production of iron-scavenging siderophores (18). In this condition, *S. aureus* should down-regulate non-essential iron containing enzymes to preserve iron for vital processes. However, despite significant research efforts devoted to the characterization of this major pathogen, no such system has been identified to date (18).

As sRNAs are major regulators of iron homeostasis in several species, we designed a selection to identify staphylococcal sRNA that could be involved in this process. We established a collection of *S. aureus* sRNA gene mutants that was used in fitness assays during iron starvation conditions. A single sRNA emerged as being essential for optimum growth when iron becomes scarce. We named it IsrR for 'iron-sparing response regulator', an acronym reflecting its function uncovered by the present study. In anaerobic conditions, expression of *isrR* prevented nitrate respiration, a non-essential metabolic pathway involving several iron-containing enzymes. IsrR is required for full lethality in an animal septicemia model of infection.

This study uncovers an iron-sparing response in *S. aureus*, dictated by a regulatory RNA, IsrR, which reallocates iron to essential processes when it is scarce.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions

Bacterial strains used for this study are described in Supplementary Table S1. Most of the work was performed with HG003, a *Staphylococcus aureus* model strain widely used for regulation studies (19). Gene annotations refer to NCTC8325 nomenclature (file CP00025.1) retrieved from Genbank and Aureowiki (20). Plasmids were engineered by Gibson assembly (21) in *Escherichia coli* IM08B (22) as described (Supplementary Table S2), using the indicated appropriate primers (Supplementary Table S3) for PCR amplifications. Plasmids expressing sGFP were constructed in MG1655Z1 *pcnB::Km*, a strain that decreases ColE1 plasmid copy number and therefore reducing toxic effects produced by high amounts of certain proteins in *E. coli*. MG1655Z1 *pcnB::Km* was constructed by the introduction of the *pcnB::Km* allele (23) in MG1655Z1 (24) by P1-mediated transduction.

Plasmids were verified by DNA sequencing and transferred into HG003, 8325-4, RN4220 or their derivatives. Chromosomal mutants (deletions and insertions) were either reported (25) or constructed for this study (Supplementary Table S1) using pIMAY derivatives as described (25), except for the *fur::tet* allele which was transferred from MJH010 (26) to HG003 and HG003  $\Delta$ *isrR::tag135* by phage-mediated transduction.

Staphylococcal strains were routinely grown in Brain Heart Infusion (BHI) broth at 37°C aerobically or anaerobically under anaerobic conditions (5% H<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>) in an anaerobic chamber (Jacomex).  $\Delta$ *fur* *S. aureus* derivatives in anaerobic condition were grown in Tryptic Soy Broth (TSB). *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. Antibiotics were added to media as needed: ampicillin 100 µg/ml and chloramphenicol 20 µg/ml for *E. coli*; chloramphenicol 5 µg/ml and kanamycin 60 µg/ml for *S. aureus*. Iron-depleted media was obtained by the addition of either DIP (2,2'-dipyridyl) 1.25 mM, unless stated otherwise; or EDDHA (ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)) 0.7 mM and incubated for 30 min prior to the addition of bacteria.

### **Fitness assay**

Mutants with altered fitness in the presence of iron chelators were identified and analyzed using a reported strategy (25) with three independent libraries containing 48 mutants (Supplementary Table S4). The libraries were grown at 37°C in BHI, BHI DIP 1.25 mM and BHI EDDHA 0.7 mM for 3 days. Overnight cultures were diluted 1000 times in fresh pre-warmed medium. Samples were withdrawn at OD<sub>600</sub> 1 and overnight as indicated (Figure 1A).

### **Spot tests**

Strain plating efficiency was visualized by spot tests. 5 µL of ten-fold serial dilutions of overnight cultures were spotted on plates containing either BHI, BHI DIP 1.25 mM or BHI EDDHA 0.7mM. Plates were supplement with chloramphenicol 5 µg/ml when strains with plasmids were tested. BHI plates were incubated overnight at 37°C while BHI DIP and BHI EDDHA were incubated for 24h.

### **Northern Blots**

Total RNA preparations and Northern blots were performed as previously described (27). 10 µg of total RNA samples were separated either by agarose (1.3%) or acrylamide (8%) gel electrophoresis. Membranes were probed with primers <sup>32</sup>P-labelled using Megaprime™ DNA labelling system (GE Healthcare) (for primers, see Supplementary Table S3) and scanned using the Amersham Typhoon imager.

### **Rifampicin assay**

Bacteria were cultured overnight in BHI. Then, 200 µl of cultures were transferred into 60 ml of fresh BHI or BHI supplemented with DIP 1.4 mM and grown at 37°C. At OD<sub>600</sub> 1.5 (t<sub>0</sub>), rifampicin was added to a final concentration of 200 µg/ml. Six ml of cultures were harvested at t<sub>0</sub> and 1, 3, 5, 10 and 20 min after addition of rifampicin and transferred to tubes in liquid nitrogen to stop bacterial growth. RNA was extracted as described (27) and Northern blots were performed as described above. tmRNA was used as RNA loading control.

## Biocomputing analysis

DNA-seq data from fitness experiments were analyzed using a reported pipeline (25). *isrR* orthologs were identified thanks to the sRNA homolog finder GLASSgo (28) with *isrR* (174nt) as input using the default parameters. Fur boxes were detected using the motif finder FIMO (29) with *isrR* sequences retrieved from GLASSgo (option including 100 nt upstream *isrR* sequences) and *S. aureus* NCTC8325 Fur consensus motifs from RegPrecise (30). IsrR secondary structure was modelled with LocARNA (31) with 18 IsrR orthologs with non-identical sequences using default parameters. Putative IsrR targets were found by CopraRNA (32) set with default parameters and *isrR* orthologs from *S. aureus* NCTC8325 (NC\_007795), *S. epidermidis* RP62A (NC\_002976), *S. lugdunensis* HKU09-01 (NC\_013893), *Staphylococcus pseudintermedius* HKU10-03 (NC\_014925), *Staphylococcus pseudintermedius* ED99 (NC\_017568), *Staphylococcus xylosus* (NZ\_CP008724), *Staphylococcus warneri* SG1 (NC\_020164), *Staphylococcus pasteurii* SP1 (NC\_022737), *Staphylococcus hyicus* (NZ\_CP008747), *Staphylococcus xylosus* (NZ\_CP007208), *Staphylococcus argenteus* (NC\_016941), *Staphylococcus carnosus* TM300 (NC\_012121), *Staphylococcus cohnii* (NZ\_LT963440), *Staphylococcus succinus* (NZ\_CP018199), *Staphylococcus agnetis* (NZ\_CP009623), *Staphylococcus piscifermentans* (NZ\_LT906447), *Staphylococcus stepanovicii* (NZ\_LT906462), *Staphylococcus equorum* (NZ\_CP013980), *Staphylococcus nepalensis* (NZ\_CP017460), *Staphylococcus lutrae* (NZ\_CP020773), *Staphylococcus muscae* (NZ\_LT906464), *Staphylococcus simulans* (NZ\_CP023497).

## Probability calculation

For a set of N objects (number of *S. aureus* mRNAs = 2889) with m different objects (number of mRNA expressing Fe-S containing proteins = 32 (estimated number in *S. aureus* [Table S6, (33,34)]), the probability of drawing n objects (23 best targets proposed by CopraRNA) and to have among k differentiated objects (mRNA expressing Fe-S containing proteins = 7) is given the following equation  $p(X = k) = \frac{C_m^k C_{N-m}^{n-k}}{C_N^n}$  where C represents a combination operator (<https://www.dcode.fr/picking-probabilities>).



### **Electrophoretic mobility shift assay**

RNAs were transcribed using the T7 RiboMAX Express Large Scale RNA Production System (Promega), purified by ethanol precipitation (for primers, see Supplementary Table S3), and then resuspended in 1X RNA binding buffer (10 mM Tris pH 8, 50 mM NaCl, 50 mM KCl and 10 mM MgCl<sub>2</sub>). Before the experiment, RNA working solutions were prepared by diluting RNAs in RNA binding buffer: 1 μM for IsrR, 25 μM for *fdhA*. RNAs were denatured individually at 90°C for 2 min and cooled for 10 min at room temperature for refolding. Different RNAs were then mixed and samples were adjusted to 15 μL with RNA binding buffer. Samples were then incubated for 15 min at 37°C to allow interactions. Three μL of 6X loading dye (non-denaturing) was added, and samples were loaded on a 6% non-denaturing acrylamide gel. Sample migration was performed for 3h at 10 V/cm. Following migration, RNAs were transferred on a Hybond membrane and then probed for IsrR (Supplementary Table S3).

### **Fur boxes reporter assay**

Fluorescent tests to evaluate the contribution of Fur boxes were performed with 8325-4, a 'pigment-less' strain (35) containing either pP<sub>IsrR</sub>::*gfp*, pP<sub>IsrR1</sub>::*gfp*, pP<sub>IsrR2</sub>::*gfp* or pP<sub>IsrR1&2</sub>::*gfp* (Supplementary Table S2). Overnight cultures were diluted 100 times, sonicated for 30 seconds and fixated with ethanol 70% and PBS 1X. Fluorescence was detected using a Partec Cube 6 flow cytometer with ≈ 500 000 cells for each measures, with 488 and 532 nm wavelength filters for excitation and emission, respectively.

### **Quantification of nitrite**

*S. aureus* cultures were grown aerobically overnight, diluted 1000 times in fresh media and grown anaerobically overnight. They were then diluted to OD<sub>600</sub> 0.01, after 2 hours of incubation at 37°C, NaNO<sub>3</sub> 20 mM was added. 150 or 240 min after, samples were recovered and nitrite in supernatant was determined using the Griess Reagent System (Promega) according to the manufacturer's instructions. OD<sub>600</sub> and OD<sub>540</sub> were determined with a microtiter plate reader (CLARIOstar).

### **5'/3' RACE mapping**

5'/3' RACE was performed as previously described (36) with some modifications. Total RNA (6 μg) from HG003 Δ*fur* was treated with TAP (Epicentre) for 1 h at 37°C. After

phenol/chloroform extraction, followed by ethanol precipitation, RNA was circularized using T4 RNA ligase (Thermo Scientific) overnight at 16°C. Circularized RNA was once more extracted with phenol/chloroform, ethanol precipitated and reverse transcribed using primer 2728 (Supplementary Table S3). A first PCR across the 5'/3' junction was performed using primers 2729/2730, followed by a nested PCR with primers 2731/2732. The PCR products were cloned using the pJET1.2/blunt system (Thermo Scientific) within *E. coli* and 20 samples were sequenced.

### **sRNA activity reporter assay**

The principle of a reporter assay for sRNA activity was described for Enterobacteria (37) and Gram-positive bacteria (38). The effect of sRNAs on targets is determined *via* the quantification of leader reporter fusions. The latter results from in frame cloning of *gfp* downstream of 5'UTRs and first codons of target genes. sRNAs and reporter genes are both on plasmids. We developed a similar system to test *IsrR* activity against its putative substrates.

Plasmids driving constitutive expression of *isrR* (pRMC2ΔR-*IsrR*) and its derivatives with CRR deletions (pRMC2ΔR-*IsrR*ΔCRR1, pRMC2ΔR-*IsrR*ΔCRR2 and pRMC2ΔR-*IsrR*ΔCRR3 expressing *isrR*ΔCRR1, *isrR*ΔCRR2 and *isrR*ΔCRR3, respectively) were constructed and introduced in HG003 Δ*isrR* (Supplementary Tables S1 and S2). These strains were subsequently transformed with p5'*FdhA*-GFP, p5'*NarG*-GFP, p5'*NasD*-GFP, p5'*GltB2*-GFP reporting the activity of *IsrR* on *fdhA*, *narG*, *nasD* and *gltB2* mRNAs, respectively (Supplementary Tables S1 and S2). The latter plasmids express the complete interaction region with *IsrR* of each mRNA target predicted by IntaRNA plus ten additional codons in frame with the sGFP coding sequence. Each gene fusion is under the control of the *sarA* promoter. Of note, the four reporter plasmids were constructed in MG1655Z1 *pcnB*::Km. Fluorescence on solid medium was visualized from overnight cultures that were streaked on plates supplemented with chloramphenicol 5 μg/ml and kanamycin 60 μg/ml. Fluorescence was detected using Amersham Typhoon scanner at 488 nm and 525 nm wavelengths for excitation and emission, respectively. Fluorescence in liquid was visualized from overnight cultures diluted 1000-fold in TSB supplemented with chloramphenicol 5 μg/ml and kanamycin 60 μg/ml, and grown in microtiter plates. Fluorescence was measured after six hours of growth using a microtiter plate reader

(CLARIOstar), normalized to OD<sub>600</sub> and set at 1 for the strains with the control plasmid (pRMC2ΔR).

### SHAPE experiments

Synthetic genes placing *isrR* and, portions of *fdhA*, *narG*, *nasD* and *gltB2* under the transcriptional control of the T7 RNA polymerase were constructed by PCR on HG003 genomic DNA using primers indicated in Supplementary Table S3. The resulting synthetic genes were *in vitro* transcribed using T7 RNA polymerase (39). RNA integrity and folding homogeneity were assessed by agarose gel electrophoresis.

RNAs were probed in the presence and absence of 5 mM Mg<sup>2+</sup> using methyl-7-nitroisatoic anhydride (1M7) and modifications were revealed by selective 2'-hydroxyl acylation and analyzed by primer extension (SHAPE) as described (40,41) with slight modifications. Briefly, 12 pmoles of RNA (*isrR*, *fdhA*, *narG*, *nasD* and *gltB2*) were diluted in 64 μl of water and denatured for 5 min at 85°C. Then, 16 μl of pre-warmed (37°C) folding buffer (final conc. 40 mM HEPES pH 7.5, 150 mM KCl with/without 5 mM MgCl<sub>2</sub>) were added and the solution was cooled down to room-temperature for 7 min. Samples were then incubated for 5 min in a dry bath set at 37°C.

Similarly, when investigating interactions, RNAs were refolded in separate tubes (12 pmoles of *IsrR* and 60 pmoles of target RNA or the opposite) as described above, then mixed and incubated for 20 min at 37°C.

Refolded RNAs were split in two and either 1M7 (final concentration of 4 mM) or DMSO was added and incubated at 37°C for 5 min. Probed RNAs were precipitated in ethanol in the presence of ammonium acetate 0.5 M, ethanol and 20 μg of glycogen. The pellets were washed twice with 70% ethanol, air-dried and resuspended in 10 μl of water.

Modifications were revealed by elongating fluorescent primers (WellRed D2 or D4 fluorophore from Sigma, for sequences see Supplementary Table S3) using MMLV reverse transcriptase RNase H Minus (Promega). Purified resuspended cDNAs were sequenced on a CEQ 8000 capillary electrophoresis sequencer (9+). Resulting traces were analyzed using QuSHAPE (42). Reactivity was obtained from at least three independent replicates. Reactivity value was set arbitrarily to -10 for the nucleotides for which it could not be determined (intrinsic RT stops). Secondary structures were

modeled using IPANEMAP (43) and drawn with VaRNA (44). Probing profiles were compared as described (39).

### **Animal studies**

Female Swiss mice (Janvier Labs), 6-8 weeks old and weighing ~30 g were used for the septicemia model. Experiments were monitored in the ARCHE-BIOSIT animal lab in Rennes, and were performed in biological duplicates. We used groups of 5 mice for the mild septicemia model. Mice were infected intravenously by the tail vein with 200  $\mu$ l of suspensions in 0.9% NaCl containing  $3 \times 10^8$  bacteria with either HG003, HG003  $\Delta$ *isrR*, or HG003  $\Delta$ *isrR* locus2::*isrR*<sup>+</sup> strains. Mouse survival was monitored for 8 days, and the statistical significance of difference(s) between groups was evaluated using the Mantel-Cox test. A *p* value < 0.05 was considered as significant. All experimental protocols were approved by the Adaptive Therapeutics Animal Care and Use Committee (APAFiS #2123–2015100214568502v4).

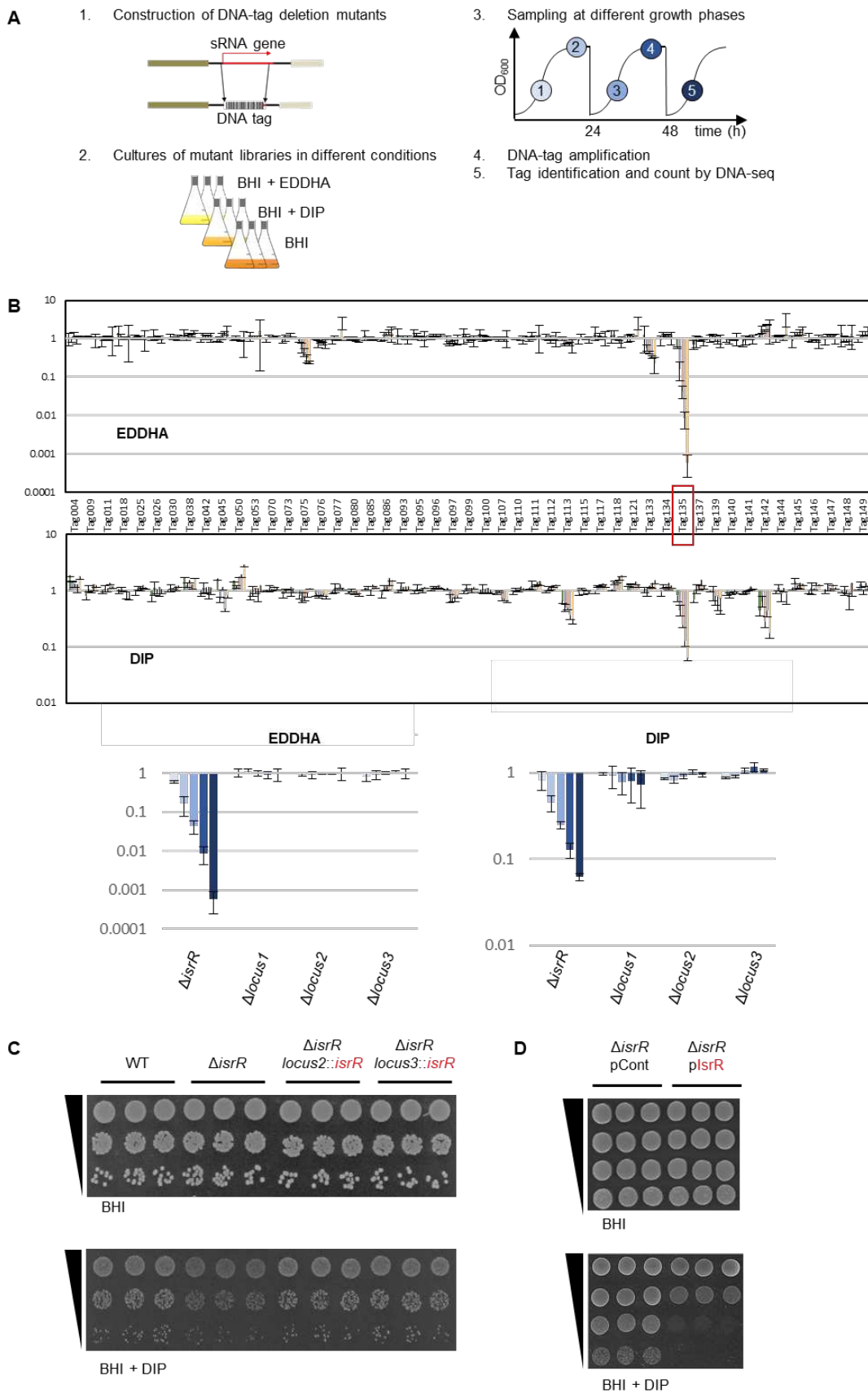
## RESULTS

### **IsrR is an sRNA required for growth in iron-depleted media.**

Iron is essential for *S. aureus* pathogenicity (1) and sRNAs are ubiquitous regulators for adaptation (45); we asked whether sRNAs are required in this bacterium to adapt to low iron availability, a condition encountered during infection. To address this question, we took advantage of a strategy that we recently developed, based on a competitive fitness evaluation of sRNA mutant libraries (25). A library of DNA-tagged deletion mutants was constructed in the HG003 strain (Supplementary Table S1); three independent mutants were constructed for each locus, to be subsequently used to constitute three independent libraries (Supplementary Table S4). Gene deletions of sRNAs corresponding to UTR regions or antisense from coding sequences more likely lead to phenotypes due to their associated coding genes; such mutations would interfere with the screening procedure designed to uncover the fine-tuning activity of sRNAs. We therefore restricted our collection to 48 mutants corresponding to nearly all known HG003 “*bona fide* sRNAs”, defined as those that are genetically independent with their own promoter and terminator (46). Deletion and substitution of these genes by tag sequences were designed to avoid interference with expression of adjacent genes. The deleted region of each mutant was replaced by a specific DNA tag, which allowed us to count each mutant and therefore to evaluate their proportion within a population of mutant strains. Using an indexed PCR primer pair, up to 40 samples can be tested in one DNA-seq run (25). Mutants that disappeared or accumulated in a given stress condition indicated a functional role of the corresponding sRNAs with respect to the imposed growth conditions.

The triplicate libraries were challenged to iron depletion by addition of iron chelators, 2,2'-dipyridyl (DIP) and ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid (EDDHA) to growth media. The proportion of each mutant within the population was determined at different growth steps over 3 days (Figure 1A). Results were normalized to the same medium without iron chelator. Among 48 tested mutants, the strain with tag135 bearing a tagged deletion between the *arlR* and *pgpB* operons had a significant fitness disadvantage with each of the two tested iron chelators (Figure 1B); after about 28

generations, its distribution compared to the control condition decreased more than 10- and 1000-fold in DIP and EDDHA, respectively.



**Figure 31. *S. aureus* IsrR sRNA is required for optimal growth when iron is scarce.**

(A) Experimental protocol scheme to select mutants with altered fitness in media containing iron chelators. (B) Evolution of mutant proportions in libraries (for composition, see Supplementary Table S4) grown in the presence of EDDHA 0.7mM or DIP 1.25mM normalized to the same libraries grown in the absence of iron chelators. Error bars indicate the standard deviation from three independent libraries. Upper parts; data with the complete 48 mutant libraries. Tag135 corresponding the *isrR* mutant is highlighted by a red box. For Tag/mutant correspondence, see Supplementary Table S1. Note that Tag145 and Tag149 are in a single strain, which corresponds to a double mutant ( $\Delta sprX2 \Delta sprX1$ ). Lower parts, selected data (enlargement):  $\Delta isrR$  and three other tagged regions are shown. Loci 1, 2 and 3 correspond to tag insertions in non-transcribed regions expected not to affect bacterial growth. Histogram color code corresponds to sampling time color code from Figure 1A. (C)  $\Delta isrR$  growth defect in iron-depleted media is complemented by an *isrR* ectopic chromosomal copy. For strain constructions see Supplementary Table S1 and Supplementary Figure S2A. Plating efficiency of indicated strains on BHI medium without (upper panels) or with (lower panels) DIP 1.25mM. Three independent biological clones are shown for each strain. For results with EDDHA, see Supplementary Figure S2C. (D) Multicopy *isrR* is toxic in iron depleted media. Experiments were performed as for Figure 1C. pCont, pCN38; plsR, pCN38-IsrR. For strain and plasmid constructions see Supplementary Tables S1 and S2.

The deletion associated with tag135 inactivated an sRNA reported in two global studies under the names S596 (47) and Tsr25 (48). It is renamed here IsrR for iron-sparing response regulator. Neither *rnalIII*, *rsaA*, *rsaC*, *rsaD*, *rsaE*, *rsaG*, *rsaOG*, *ssr42* nor *ssr* deletion mutants (46) present in the tested libraries were impacted by the presence of either DIP or EDDHA, indicating that their corresponding sRNAs do not significantly contribute to adaptation to low iron conditions in the tested conditions.

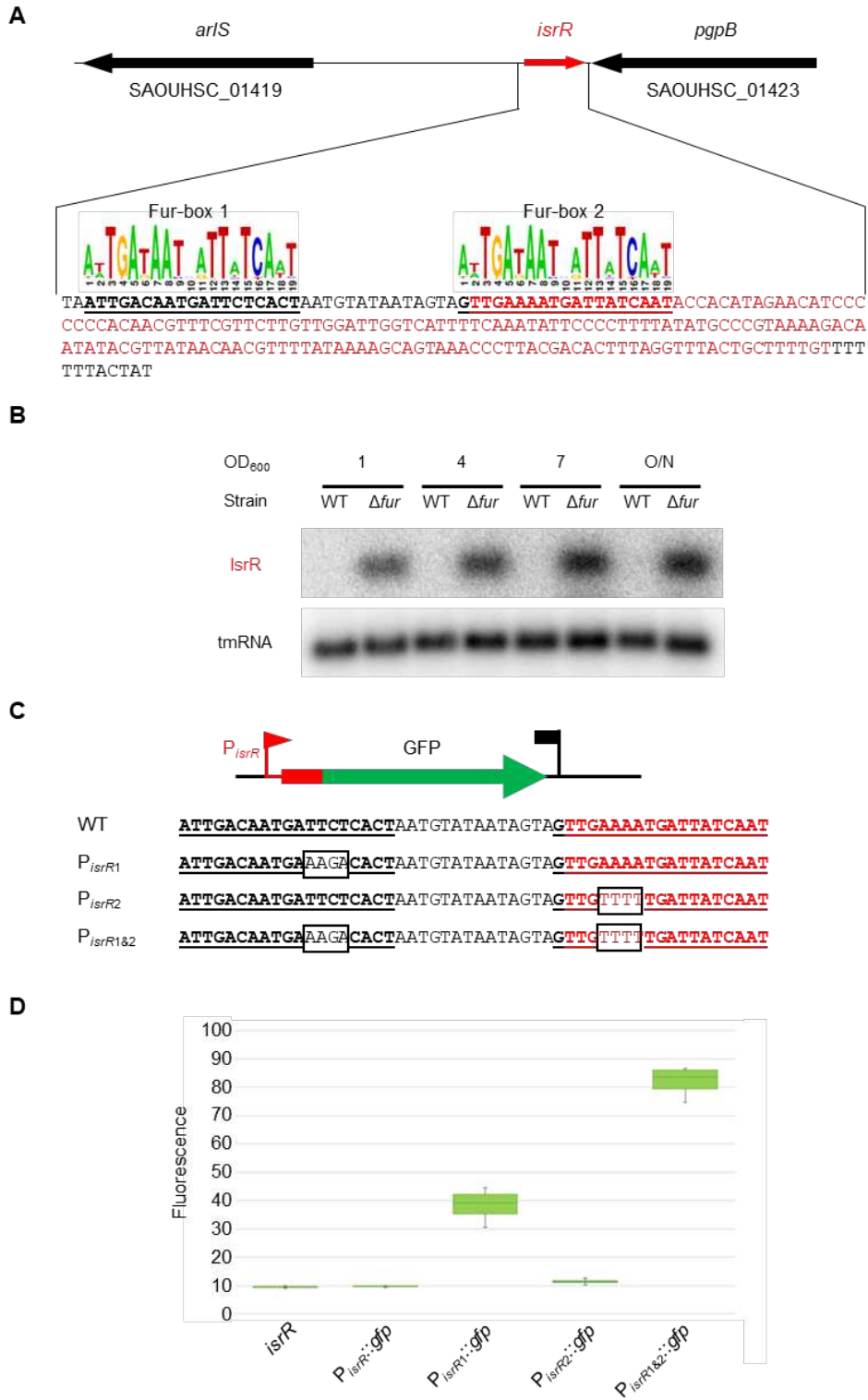
The HG003  $\Delta isrR$  strain had no apparent growth defect in rich medium compared to its parental strain (Figure 1C and Supplementary Figure S1). However, by spot tests, a  $\Delta isrR$  mutant displayed reduced colony-size in the presence of DIP and EDDHA, supporting the fitness experiment results and evidencing that the individual  $\Delta isrR$  mutant, in the absence of the other 47 mutants, is required for optimal growth when the iron supply is limited (Figure 1C and Supplementary Figure S1). Of note, the slight growth retardation of  $\Delta isrR$  observed on iron-depleted plates or in liquid cultures corresponds to a drastic fitness cost when in competition with other bacteria. To confirm that the phenotype was solely due to the absence of IsrR, a copy of *isrR* with its endogenous promoter was inserted in the chromosome of the  $\Delta isrR$  strain at two different loci, leading to either  $\Delta isrR$  locus2::*isrR*<sup>+</sup> or  $\Delta isrR$  locus3::*isrR*<sup>+</sup> strains (Supplementary Figure S2A). Loci 2 and 3 were chosen since these regions were located between two terminators and seemed not to be transcribed. *isrR* expression from *loci* 2 and 3 was confirmed by Northern blot (Supplementary Figure S2B). The two  $\Delta isrR$  strains carrying a single copy of the *isrR* gene had plating efficiencies equivalent to that of the isogenic parental strain when grown with DIP or EDDHA, with colony sizes similar to those of the parental strain. These results

confirm that the observed  $\Delta isrR$  phenotype was strictly dependent on *isrR* (Figure 1C and Supplementary Figure S2C). Of note, HG003 carrying *pIsrR* (*IsrR* expressed from its endogenous promoter) leads to a 100-fold reduced ability to form colonies on iron-depleted media compared to the strain carrying a control vector (Figure 1D). Multicopy *isrR* expression is deleterious for *S. aureus*, possibly due to the disruption of important metabolic pathways; thus, we cannot rule out that the effect is due to an *IsrR* off-target activity. We conclude that the absence of *IsrR* is detrimental to *S. aureus* growth under iron-depleted conditions.

### ***IsrR* expression is repressed by the ferric uptake regulator Fur.**

Extremities of *IsrR* were determined by 5'/3'RACE experiments after circularization of total RNAs (Supplementary Figure S3A). *isrR* transcription starts at position 1362894 and terminates at position 1363067 on the NCTC8325 genome map, generating a 174 nucleotide-long sRNA (Figure 2A). *isrR* is preceded by a  $\sigma^A$ -dependent promoter and ends with a rho-independent terminator (47). Its high expression in a chemically-defined medium and the presence of a putative Fur-box within the promoter region suggested iron-dependent expression of *isrR* (47). Fur-dependence of *isrR* regulation was verified in a HG003  $\Delta fur$  mutant (Supplementary Table S1). In rich medium, *IsrR* was not detected by Northern blotting in the parental strain but strongly accumulated in the  $\Delta fur$  mutant, demonstrating that Fur negatively controls *isrR* expression (Figure 2B). The *isrR* gene is predicted to be controlled by two Fur-boxes, one within the promoter region and the second immediately downstream of the transcriptional start site (Figure 2A). To test their relative contributions, a plasmid-based transcriptional reporter system placing a *gfp* gene under control of the *isrR* promoter was constructed ( $P_{isrR}::gfp$ ) (Supplementary Table S2). The contribution of each predicted Fur box was tested by targeted mutagenesis altering either the first ( $P_{isrR1}::gfp$ ), the second ( $P_{isrR2}::gfp$ ) or both ( $P_{isrR1\&2}::gfp$ ) Fur-binding motifs (Figure 2C). As expected, the transcriptional reporter generated no fluorescence in a *fur*<sup>+</sup> strain (Figure 2D). Alteration of the upstream box led to strong fluorescence, which was even more intense when both boxes were mutated. In contrast, mutations only in the downstream Fur motif had no effect on the expression of the reporter fusion. We concluded that both sites contribute to efficient *isrR* Fur-dependent repression, the first one being epistatic over the second.





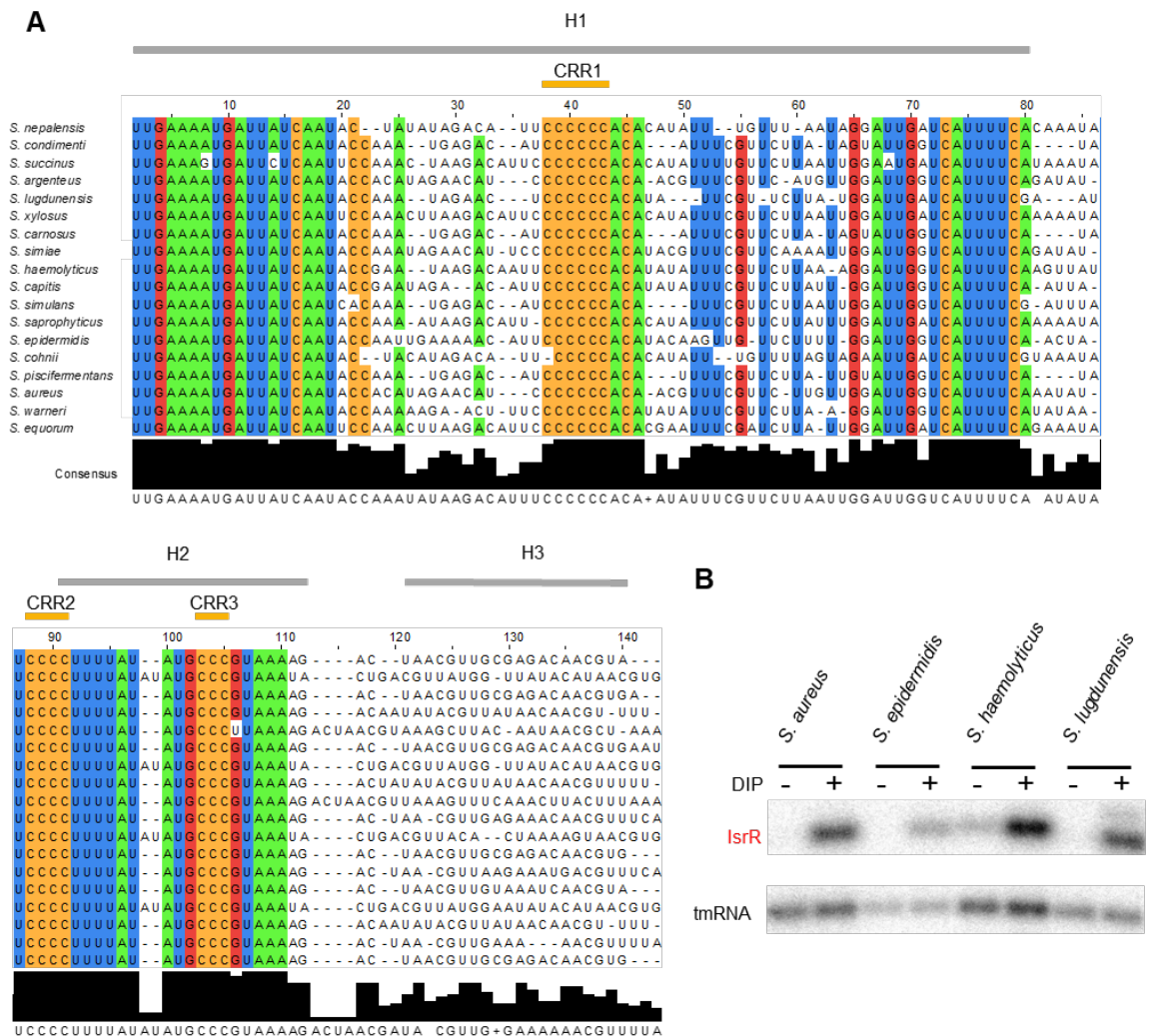
**Figure 32. IsrR is regulated by Fur.**

(A) *isrR* genetic locus. The transcribed region is in red. For *isrR* 3'-5'RACE mapping results, see Supplementary Figure S3A. Predicted Fur boxes are bold underlined. The staphylococcal Fur box consensus is taken from the RegPrecise database (30). (B) Northern blot experiment with HG003 and its *fur* derivative sampled at the indicated OD<sub>600</sub> and probed for IsrR and tmRNA (control) (n=2). (C) *isrR* Fur box sequence (WT) and its mutant derivatives ( $P_{isrR1}$ ,  $P_{isrR2}$  and  $P_{isrR1\&2}$ ). Mutations altering the Fur boxes are shown in black boxes. (D) Fluorescence of transcriptional fusions placing *gfp* under the control of the

*isrR* promoter and its mutant derivatives (as indicated) measured by flow cytometry, (n=5). The first strain indicated '*isrR*' is a control strain in which the *isrR* gene is not fused to *gfp*.

### IsrR and its regulation by Fur are conserved in the *Staphylococcus* genus.

Most *trans*-acting bacterial sRNAs are poorly conserved across species (49,50), including among the staphylococci (46,51). However, the *isrR* gene was detected in all screened genomes from the *Staphylococcus* genus (Figure 3A and Supplementary Table S5). Sequence conservation includes the two Fur boxes, which were detected for all *isrR* gene sequences of the different *Staphylococcus* species.



**Figure 33. IsrR is conserved and Fur-regulated within the *Staphylococcus* genus.**

(A) Sequence conservation. The alignment was obtained using LocARNA (31) with *isrR* sequences from indicated strains generated by GLASSgo (28) as inputs. 80% conserved nucleotides within the tested sequences are colored (green, A; blue, U; red, G; other, C). Sequences shown are limited to the three first stem-loops (H1 to H3). There is poor nucleotide sequence conservation for H3 and the transcriptional terminator. Three C-rich regions are indicated (CRR1 to CRR3). (B) Northern blot probed for IsrR in *S. aureus* HG003, *S. epidermidis* ATCC 12228, *S. haemolyticus* JCSC1435, and *S. lugdunensis* N920143. Bacteria were grown in BHI or BHI supplemented with DIP 1.25 mM and were withdrawn at OD<sub>600</sub> 1 (n=2).

IsrR orthologs from different species of the *Staphylococcus* genus were used to feed LocARNA, a software for multiple alignments of RNAs that generates secondary structure predictions and highlights conserved pairing regions (31). The proposed IsrR structure includes three stem-loops (H1 to 3) and a canonical Rho-independent terminator (T) (Supplementary Figure S3B). Three conserved C-rich regions, named here CRR1 to 3, are predicted to be single stranded. CRR1 and 3 are within the loops of stem loop structures H1 and H2, while CRR2 is at the 5'-end abutting stem H2. CRR1 is four to seven C-long, according to the species considered. C-rich motifs are important for sRNA/target recognition in *S. aureus* (52-54). This feature efficiently discriminates targets in staphylococcal strains, since their genomes are ~70% AT-rich, and G-rich stretches are infrequent except within Shine-Dalgarno sequences. Two first stems, H1 and H2, have conserved primary sequences with little covariation, indicating the importance of each nucleotide, possibly for an interaction with a yet unknown protein. Of note, single-stranded regions adjacent to CRR and within the loops are more variable than H1 and H2. IsrR was subjected to SHAPE chemical probing, a technology revealing single-stranded nucleotides. RNAs were incubated with 1-Methyl-7-nitroisatoic anhydride (1M7), which specifically reacts with flexible nucleotides (55,56). Reactivity data was implemented into the IPANEMAP workflow to predict IsrR secondary structure according to thermodynamics and probabilistic calculations balanced with experimental data (43). The model obtained (Supplementary Figure S4) is identical to the one obtained using the phylogenetic approach described above. Most nucleotides with high reactivity were identified in predicted single-stranded regions. However, several nucleotides with medium to high reactivity are in predicted double stranded regions probably reflecting the poor stability of such A-U rich structures. Surprisingly, CRR1 is weakly reactive, and CRR2 and CRR3 are only mildly reactive, suggesting that although modeled in single-stranded regions, these Cs are involved in some interactions. As IsrR does not feature any stretches of G, Cs from the CRRs might be involved in yet to be identified non-canonical interactions. Of note, such interactions could actually expose the C's Watson-Crick face to the solvent making them available for an intermolecular interaction. To detect divalent ion-dependent tertiary folding, IsrR was probed in the presence or absence of Mg<sup>2+</sup> ions (Supplementary Figure S4). Only very few significant

local reactivity changes were observed suggesting that IsrR does not adopt an extensive tertiary structure (57).

The expression and iron dependency of IsrR were experimentally confirmed for *S. haemolyticus*, *S. epidermidis* and *S. lugdunensis* (Figure 3B). Our results suggest that IsrR has an important function related to iron metabolism that is conserved throughout the *Staphylococcus* genus. The presence of CRRs suggests that IsrR is likely a conserved *trans*-acting small regulatory RNA.

### **Bioinformatics predictions indicate that IsrR targets mRNAs encoding enzymes with Fe-S clusters.**

As *trans*-acting bacterial sRNAs base-pair to RNAs, it is possible to predict their putative targets by bioinformatics; however, some predictive programs generate false positive candidates (58). We used CopraRNA, a comparative prediction algorithm for small RNA targets, which takes into account RNA sequence accessibility within sRNA structures and the evolutionary conservation of the pairings (32). It is reportedly an efficient sRNA-target predictor when ortholog sRNA sequences are detected among different species, especially when they are phylogenetically distant (58). As IsrR is a conserved sRNA in the *Staphylococcus* genus, IsrR ortholog sequences were used as input for CopraRNA. Results indicated a functional convergence of the proposed targets (Supplementary Figure S5). Among the 23 top candidates with the best e-values on pairings, seven are mRNAs that encode iron-sulfur (Fe-S)-containing proteins (NasD, MiaB, GltB2, FdhA, CitB, NarG and SAOUHSC\_01062). Some of these IsrR putative targets were previously discussed (47). Considering 32 different Fe-S containing proteins in *S. aureus* identified by manually curated *in silico* analyses (detailed in Supplementary Table S6), the probability of identifying seven mRNAs encoding Fe-S containing proteins by chance is  $2 \times 10^{-9}$  (see Methods). This remarkably low probability allows one to state with high confidence that Copra RNA identified relevant targets. Other putative targets, such as *moaD* and *nreC* mRNAs, are also associated with Fe-S containing complexes (59,60). It is therefore likely that most, if not all, predicted targets associated with Fe-S clusters are direct IsrR targets. Remarkably, nitrate reductase, nitrite reductase, glutamate synthase, formate dehydrogenase, aconitate and hydratase methionine sulfoxide reductase, which are all putatively affected by IsrR (Supplementary Figure S5), also correspond to

either putative or demonstrated enzymes affected by RyhB (5,61) suggesting a striking functional convergence between these two sRNAs.

#### **IsrR targets mRNA encoding nitrate-related enzymes.**

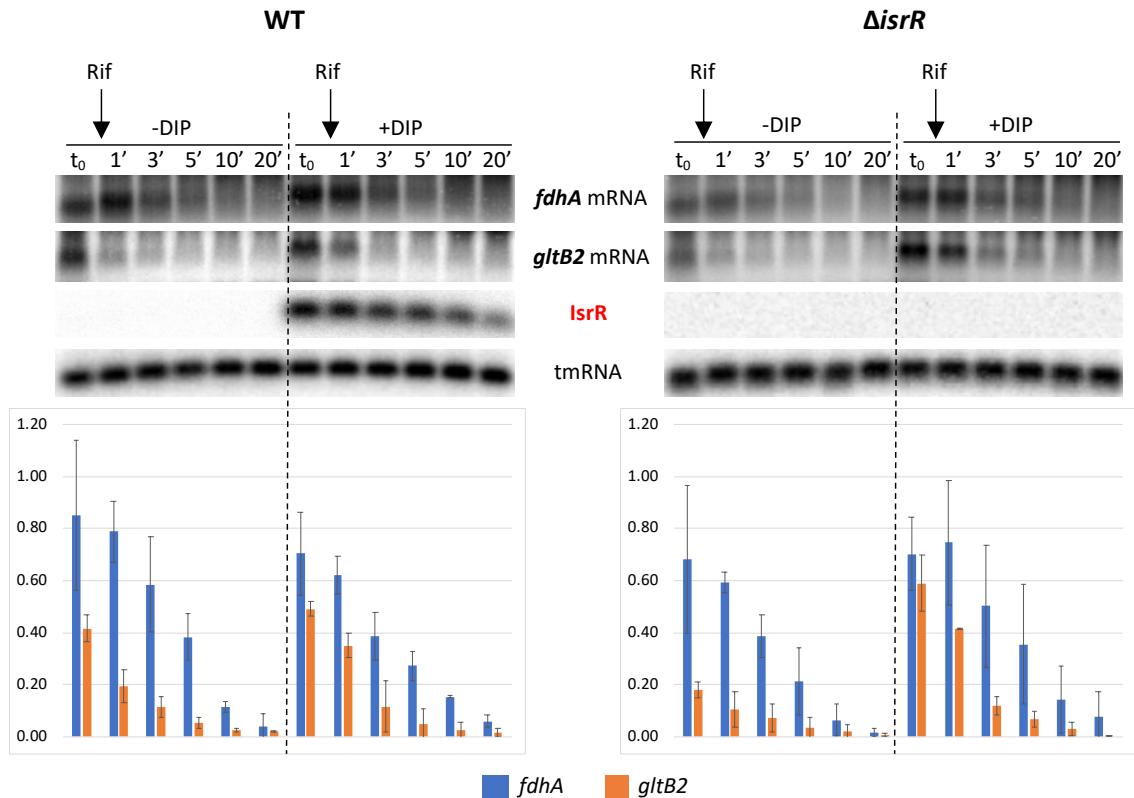
In the absence of oxygen, *S. aureus* uses nitrate, if available, as an electron acceptor of the respiratory chain (62). Nitrate is converted to nitrite by nitrate reductase encoded by the *nar* operon (Supplementary Figure S6A). In a second step, nitrite is converted to ammonium by nitrite reductase encoded by the *nas* operon. Ammonium is then used by glutamate synthase encoded by the *gltB2* gene. Transcription of *nar* and *nas* operons depends on the NreABC regulatory system, whose activity requires the presence of nitrate and the absence of oxygen (60). Strikingly, *narG* (nitrate reductase subunit  $\alpha$ ), *nasD* (nitrite reductase large subunit), and *gltB2* mRNAs are IsrR putative targets, suggesting that IsrR could affect all steps of nitrate to glutamate conversion.

In *E. coli*, the *fdhF* gene encoding a molybdenum-dependent formate dehydrogenase is regulated by nitrate (63). A strain with a mutated *fdhF* allele lacks nitrate reductase activity (64), suggesting that FdhF is associated with the nitrate dissimilatory pathway. Interestingly, the *fdhF* ortholog in *S. aureus*, *fdhA*, is also predicted to be an IsrR target.

To assess IsrR targets associated with the nitrate reduction pathway, we selected the *fdhA*, *narG*, *nasD* and *gltB2* mRNAs. These mRNAs are predicted to pair with IsrR with hybridization energies ranging from -39 to -30 kcal/mol (Supplementary Figure S6B). For each, the pairing regions cover the Shine-Dalgarno sequences from the mRNAs and CRRs from IsrR, suggesting that all four mRNAs are *bona fide* IsrR targets.

Detection of long transcripts by Northern blot is often not possible in *S. aureus*. For this reason, the effects of IsrR on the amount of its targets was tested by Northern blots solely for *fdhA* and *gltB2* mRNAs, which are expressed from short operons (bi- and mono-cistronic, respectively). In many cases, the interaction of an sRNA with its mRNA target results in the destabilization of transcripts. Consequently, we questioned whether IsrR would affect *fdhA* and *gltB2* mRNA stability. RNA stability was evaluated upon transcription inhibition by rifampicin, where the variations in RNA quantities are assumed to reflect their degradation. This classical approach should nevertheless be interpreted cautiously since i) stability of sRNA and mRNA targets depends on their base

pairing and that ii) rifampicin prevents the synthesis of both partners, one of them being possibly limiting (65). In HG003 and its  $\Delta isrR$  derivative, *fdhA* and *gltB2* mRNAs were unstable in rich medium. Addition of DIP to the growth media resulted in a minor increase of both mRNAs in HG003 and  $\Delta isrR$  strain (Figure 4). However, IsrR does not have a significant effect on *fdhA* and *gltB2* mRNA stability, with the caveat associated with possible limitation of using rifampicin to assess regulatory RNA activities.



**Figure 34. Stability of *fdhA* and *gltB2* mRNAs is not significantly affected by *IsrR*.**

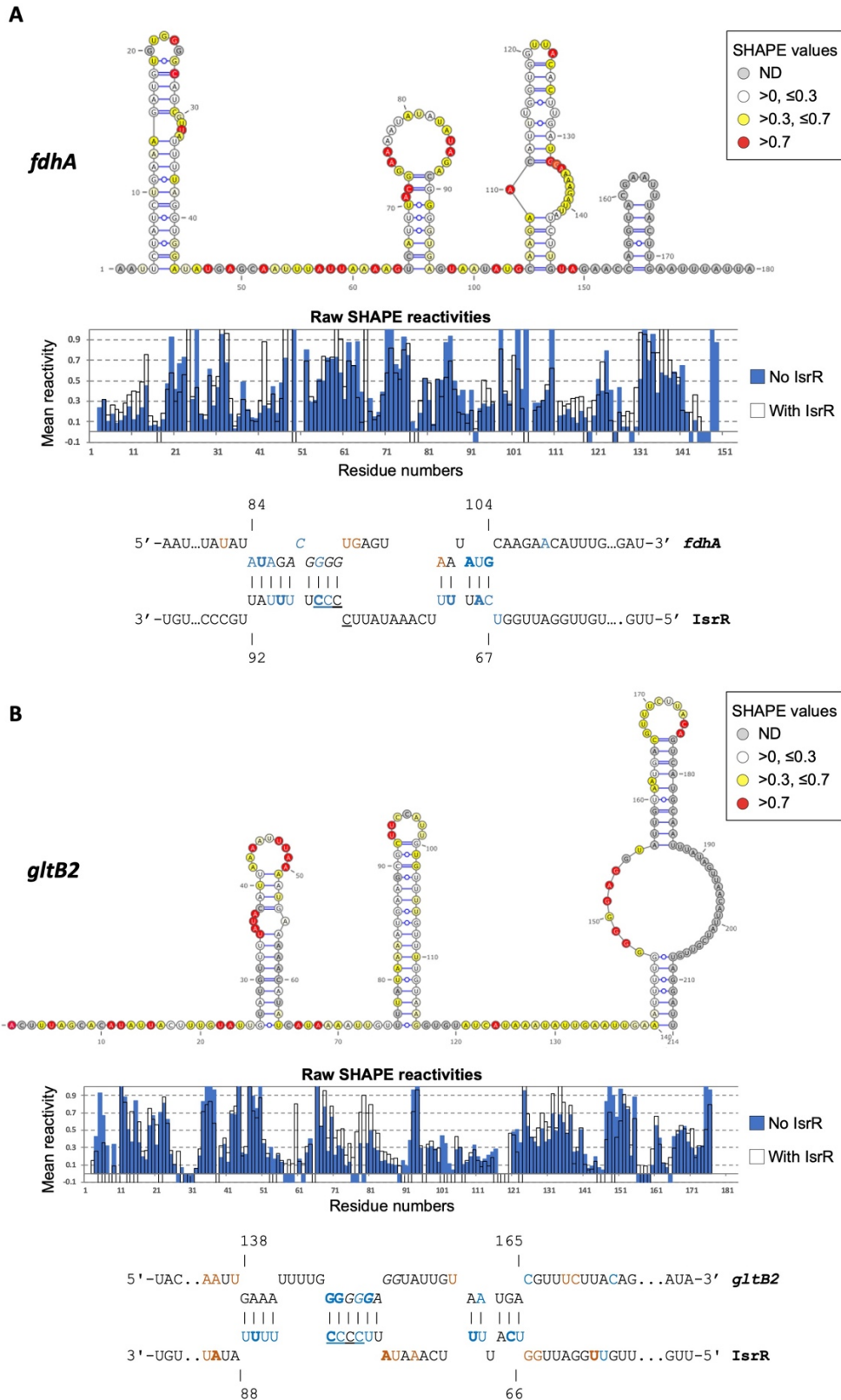
HG003 (left panel) and its isogenic  $\Delta isrR$  derivative (right panel) were grown in rich medium with or without addition of DIP (as indicated). At t<sub>0</sub>, rifampicin (Rif) was added to the growth medium. Cultures were sampled at t<sub>0</sub>, 1, 3, 5, 10 and 20 min after addition of rifampicin, total RNA was extracted, and the amounts of *fdhA* mRNA, *gltB2* mRNA, tmRNA (loading control), and *IsrR* were assessed by Northern blot. Histograms show the quantification of *fdhA* and *gltB2* mRNAs from two rifampicin assays as shown in the upper panel. Vertical axis, arbitrary units. Error bars indicate the standard deviation from two independent experiments (n=2).

Binding of *IsrR* to one putative nitrate-related targets, *fdhA* mRNA, was tested by electrophoretic mobility shift assay (EMSA). An *IsrR* band-shift was observed in the presence of *fdhA* mRNA, giving support for the formation of an *IsrR/fdhA* mRNA complex (Supplementary Figure S7). To identify nucleotides contributing to the complex formation, EMSA was performed with *IsrR* and an *fdhA* mRNA harboring point mutations in the predicted interaction zone. Surprisingly, the presence of 5 point mutations within

the *IsrR* or *fdhA* mRNA did not prevent the band shift (Supplementary Figure S7). The interaction between *IsrR* and *fdhA* mRNA is predicted to include 41 base-pairs, therefore, replacement of five nucleotides within the *IsrR* sequence is not sufficient to disrupt it.

We used the SHAPE technology to further model the targeted 5'UTR secondary structures and probe the binding between *IsrR* and nitrate-related mRNA targets, either on each RNA alone or when incubated as an sRNA / mRNA pair. IPANEMAP workflow yielded secondary structure models very consistent with the SHAPE probing data for *fdhA*, *gltB2* (Figure 5) and *nasD* (Supplementary Figure S8) mRNAs, whereas the *narG* mRNA reactivity map did not allow modelling of a stable structure, suggesting it does not adopt one. Interestingly, *fdhA* Shine-Dalgarno sequence is embedded in a stable structure whereas the initiation codon is highly reactive while the opposite is observed for *gltB2* mRNA. Unavailability of either of these sequence elements suggests that expression of these two genes is regulated by their internal structure.

Statistically significant reactivity variations for specific nucleotides were observed for *IsrR* and each of its mRNA targets when incubated alone or as an *IsrR*/5' UTR target pair (Figure 5 and Supplementary Figure S8). Such data strongly supports an interaction of *IsrR* with the four putative mRNA targets tested. Nucleotides for which reactivity decreases are likely to be involved in base pairing between *IsrR* and a target mRNA, whereas an increase of reactivity probably reflects the destabilization of a structure upon formation of the sRNA/mRNA heterodimer (*e.g.*, the initiation codon of *fdhA* and the region including the Shine-Dalgarno of *gltB2*, which were predicted to be highly reactive, presented a significant decreased reactivity in the presence of *IsrR*). These data led us to propose models for the interaction between *IsrR* and *fdhA*, *gltB2* and *nasD* mRNAs that slightly differ from those obtained by IntaRNA. The interactions we propose include *IsrR* CRR2, and the Shine-Dalgarno as well as the AUG initiator codon on the mRNA target side, suggesting that *IsrR* impairs translation of its targets.



**Figure 5. Comparison of *fdhA* and *gltB2* mRNA reactivity in the presence or absence of IsrR with proposed alternative interaction model.**

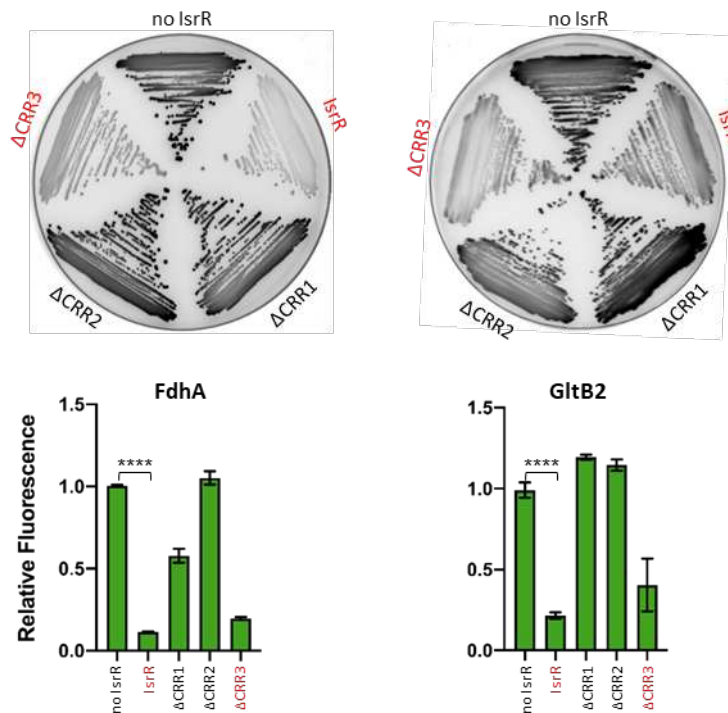
Top panels: Model obtained with IPANEMAP for *fdhA* (A) and *gltB2* (B) 5'UTRs using 1M7 reactivity as constraints. Both mRNAs were probed with 1M7 at 37°C. Nucleotides are colored according to their reactivity in the absence of IsrR with indicated color code. ND, not determined. Middle panels: average reactivity of each nucleotide within the mRNA molecule in the presence (white bars) or absence (blue



bars) of IsrR. Bottom panels: alternative model for interaction between target mRNA and IsrR based on changes in reactivity in the presence of IsrR. Nucleotides in blue, decreased reactivity, nucleotides in brown, increased reactivity. Nucleotides colored and in bold present statistically significant changes in reactivity in the presence of IsrR ( $p$  value  $<0.05$ ,  $n=3$ ). mRNA Shine-Dalgarno sequence is shown in italics; the IsrR CRR2 region is underlined.

### **Translational control by IsrR**

The presence of CRRs, pairing predictions and SHAPE results together suggest that IsrR affects translation of the selected mRNA targets. To investigate IsrR-mRNA interactions and determine the implication of IsrR CRRs in target regulation, 5'UTR sequences of each predicted mRNA target of IsrR were fused to a reporter gene. This setup further allowed us to validate CRR interactions of IsrR with *fdhA*, *narG*, *nasD* and *gltB2* mRNAs. For these experiments, sequences corresponding to the 5'UTRs and the first codons of each of the three mRNA targets were cloned under the control of the P1 *sarA* promoter in frame with the GFP coding sequence (p5'FdhA-GFP, p5'NarG-GFP, p5'NasD-GFP, p5'GltB2-GFP; Supplementary Figure S9A). The cloned regions comprise the predicted pairing domains with IsrR. The *isrR* gene was placed under the control of the P<sub>tet</sub> promoter on a multicopy plasmid (pRMC2ΔR-IsrR), and mutants lacking either the first, second or third C-rich motif were constructed, leading to pRMC2ΔR-IsrRΔCRR1, pRMC2ΔR-IsrRΔCRR2 and pRMC2ΔR-IsrRΔCRR3, respectively (Supplementary Table S1). The Δ*isrR* strains containing the different reporters were transformed with pRMC2ΔR, pRMC2ΔR-IsrR and its derivatives. We confirmed that *isrR* and its ΔCRR derivatives were constitutively expressed (Supplementary Figure S9B). For each strain containing one of the four reporter genes, expression of IsrR led to reduced fluorescence (Figure 6 and Supplementary Figure S10).



**Figure 6. Translational down-regulation by IsrR and the CRR contribution**

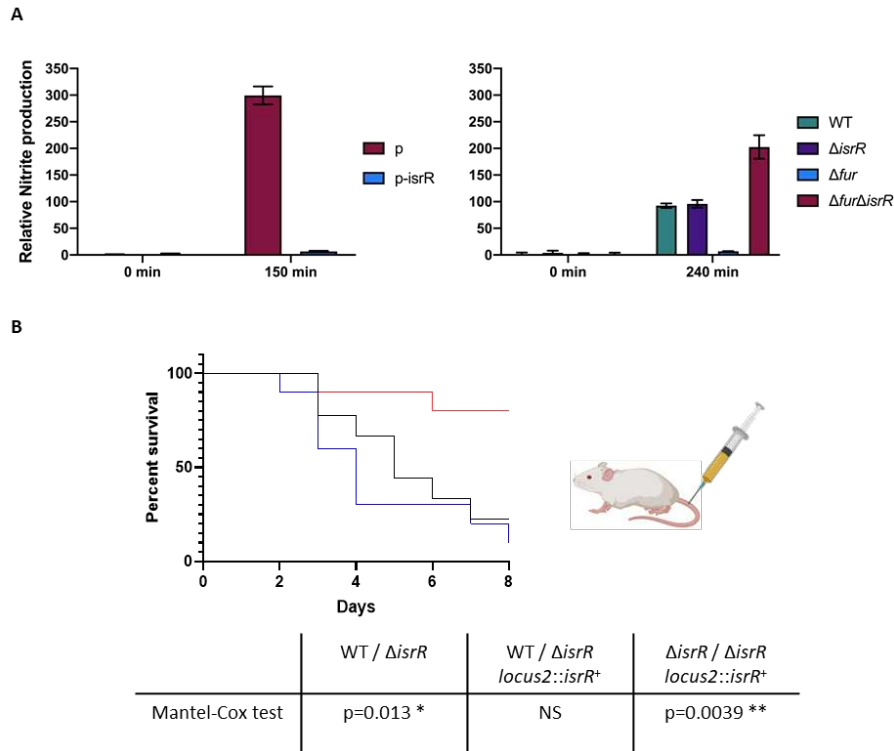
Leader fusions between the first codons of *fdhA* or *gltB2* and GFP were constructed (Supplementary Figure S9 and Table S2). The cloned fragments include the interaction regions with IsrR as described (Supplementary Figure S5). HG003  $\Delta isrR$  derivatives with either a control plasmid (no IsrR; pRMC2 $\Delta$ R), or plasmids expressing IsrR (pRMC2 $\Delta$ R-*isrR*), IsrR $\Delta$ CRR1 (pRMC2 $\Delta$ R-*isrR* $\Delta$ CRR1), IsrR $\Delta$ CRR2 (pRMC2 $\Delta$ R-*isrR* $\Delta$ CRR2), IsrR $\Delta$ CRR3 (pRMC2 $\Delta$ R-*isrR* $\Delta$ CRR3) were transformed with each engineered reporter gene fusion. Translational activity from the reporters in the presence of the different *isrR* derivatives were evaluated by fluorescence scanning of streaked clones on plates (n=3). Fully active IsrR derivatives are shown in red. Translational activity of the reporter genes with the different *isrR* derivatives was also determined in liquid culture. Fluorescence of the strains was measured in 6 h cultures using a microtiter plate reader. Results are normalized to 1 for each fusion with the control plasmid. Error bars indicate the standard deviation from three independent experiments (n=3). Statistical analysis was performed using Student t test; \*\*\*\* represents P value <0.0001.

The integrity of CRR1 was required for the IsrR activity against *fdhA* and *gltB2* reporter fusions. However, CRR1, despite being the largest CRR, was dispensable for IsrR activity against *narG* and *nasD* reporter fusions (Supplementary Figure S10). Interestingly, CRR2 was necessary for IsrR activity against all four reporter fusions. The observations are supported by SHAPE data. The integrity of at least two IsrR CRRs was required for activity against the four targets. These observations revealed that all CRRs are needed to mount the complete IsrR response, but differentially affect activity according to the given mRNA target.

#### **IsrR down-regulates nitrate metabolism.**

To determine if IsrR could indeed interfere with nitrate respiration, the amount of nitrite in anaerobic cultures upon addition of nitrate was measured in strains with either no

IsrR or overexpressing IsrR. Two systems were used: i)  $\Delta isrR$  carrying a plasmid control (pCont) compared to the same strain with a plasmid expressing IsrR (p-IsrR) and ii)  $\Delta fur$  compared to  $\Delta fur \Delta isrR$  strains; note that *isrR* is constitutively expressed in the  $\Delta fur$  background (Figure 7A). In both systems, IsrR accumulation prevented nitrite production, as expected if nitrate reductase (*narG*) is indeed down-regulated by IsrR.



**Figure 7. IsrR controls the nitrate respiration pathway and is required for virulence**

(A) IsrR prevents nitrate conversion to nitrite. Strains were grown in anaerobic conditions for two hours and nitrate (20 mM) was added to cultures. Growth media: left panel, BHI; right panel, TSB. TSB medium was used here since we observed that  $\Delta fur$  mutants grow poorly in BHI under anaerobic conditions. Samples for nitrite measurement were withdrawn at times 0 and 150 (left panel) or 240 min (right panel) upon nitrate addition. p, pRMC2 $\Delta$ R; p-IsrR, pRMC2 $\Delta$ R-IsrR. Histograms represent the relative nitrite concentration (Griess assay, OD<sub>540</sub>) normalized to the bacterial mass (OD<sub>600</sub>). Results are normalized to 1 for  $\Delta isrR$  p (left panel) and WT (right panel) samples prior to nitrate addition. Error bars indicate the standard deviation from three independent experiments (n=3). Statistical analysis was performed using Student t test (parametric unpaired); \*\*\*\* represents P value <0.0001; \*\*\* represents P value =0.0001. (B) Kaplan-Meier survival probability plots in a septicemia model of mice infected with either HG003 (WT, black), HG003  $\Delta isrR$  (red) and HG003  $\Delta isrR$  *locus2::isrR*<sup>+</sup> ( $\Delta isrR$  complemented, blue). Survival was monitored for 8 days post infection. Results shown are from 10 mice per group; the experiment was performed twice and data combined. The Mantel-Cox test was used to determine p values. NS, non-significant.

**IsrR activity is Hfq independent**

In Enterobacteriaceae, Hfq is a RNA chaperone required for sRNA-mediated regulations (66). However, it does not seem to be the case in *S. aureus* (67-69), nor for *B. subtilis* (70-72) where its function remains enigmatic. Since RyhB activity is Hfq-dependent in *E.*

*coli* (8), we asked whether IsrR would require a functional Hfq in *S. aureus*. The *fdhA*, *nasD*, *narG* and *gltB2* translation reporter genes described above were introduced into  $\Delta hfq$  strains containing either a control plasmid or a plasmid constitutively expressing IsrR. As with the parental strain (Figure 6 and Supplementary Figure S10), the reporter fusions were down-regulated by IsrR despite the absence of Hfq (Supplementary Figure S11A). It was conceivable that *isrR* cloned on a multi-copy plasmid produced high levels of sRNA that would bypass the need for Hfq. We therefore introduced the *fdhA* translation reporter in HG003, HG003  $\Delta hfq$  and HG003  $\Delta isrR$ . When present, *isrR* expression was induced from its endogenous locus by addition of DIP to the medium. On DIP-containing plates, the reporter fusion showed a reduced activity in the parental and  $\Delta hfq$  strain compared to the strain lacking IsrR (Supplementary Figure S11B). Additionally, *in vivo* nitrite production in anaerobiosis was compared qualitatively between a HG003 WT strain and its  $\Delta hfq$  derivative containing either a control plasmid or a plasmid constitutively expressing IsrR. 150 minutes after addition of nitrate ( $\text{NaNO}_3$ ), the  $\Delta hfq$  strain expressing IsrR efficiently inhibited nitrite production as observed with the parental strain (Figure S11C). Of note, IsrR induction through addition of DIP was not used in this case since under anaerobic conditions, iron starvation results in severe growth arrest of the strains. We conclude that IsrR activity does not require Hfq for the tested phenotypes.

#### **IsrR RNA is required for *S. aureus* virulence.**

Host iron scavenging plays a crucial role in *S. aureus* infection (73). As the  $\Delta isrR$  mutant has altered fitness in iron-restricted environments, we postulated that it could also impact virulence. We compared virulence of  $\Delta isrR$ ,  $\Delta isrR$  *locus2::isrR*<sup>+</sup> and parental (HG003) strains injected intravenously in a mouse septicemia model (74). Of note, the absence of *isrR* does not alter *in vitro* growth in rich media (Figure 1 and Supplementary Figure S1) and IsrR is expressed in the  $\Delta isrR$  *locus2::isrR*<sup>+</sup> strain (Supplementary Figure S2B). Most mice inoculated with strains expressing a functional *isrR* (HG003 and  $\Delta isrR$  *locus2::isrR*) were dead within 8 days (Figure 7B). In striking contrast, the lack of IsrR expression significantly reduced HG003 virulence, demonstrating the important role of this regulatory RNA during *S. aureus* infection in this model.

## DISCUSSION

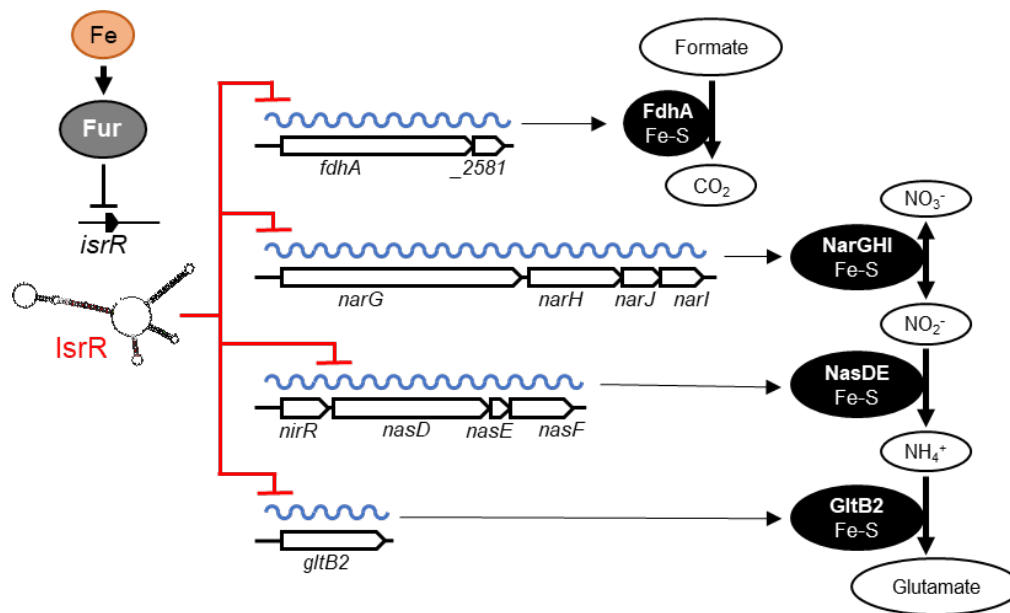
The link between *S. aureus* pathogenicity and its iron status is established (75). The host exerts nutritional immunity that depletes iron in response to invading bacteria as a means to restrict infection. Here, we identified IsrR as the only sRNA, from 45 tested, required for optimum *S. aureus* growth in iron-restricted conditions and indeed, its absence attenuates *S. aureus* virulence.

IsrR was previously reported in a large-scale transcriptome study performed in numerous growth conditions (47), and was also observed to be the most highly upregulated sRNA when *S. aureus* growth in human serum was compared to that in a rich laboratory medium (48). Environments that failed to provide free iron, such as serum, promoted the induction of iron-repressed genes in infecting bacteria (76,77). Our findings on *isrR* regulation identify iron starvation as the signal for IsrR induction. RsaOG, RsaG, Teg16, SsrS and RsaD sRNAs were reported as upregulated in serum (48), but they do not contribute to *S. aureus* optimized fitness in the iron-starved conditions we tested. Supporting our observation, their corresponding genes are not preceded by Fur boxes (46) and their susceptibility to serum is likely not related to iron starvation. RsaC is an *S. aureus* sRNA produced in response to manganese (Mn) starvation that inhibits the synthesis the superoxide dismutase SodA, a Mn-containing enzyme (78). RsaC is possibly the Mn counterpart of IsrR for iron and may contribute to adaptation to metal-dependent nutritional immunity. However, while putative RsaC targets are also associated with iron homeostasis, the *rsaC* mutant was not significantly affected by the chelating conditions tested here. IsrR is the only sRNA tested affected by both DIP and EDDHA and it is Fur regulated.

Inactivation of the Fur repressor leads to down-regulation of numerous genes (79,80). This paradoxical regulation could be achieved by a Fur-dependent expression of negative regulators, which act as 'inverters' of the Fur response, with IsrR being an *S. aureus* Fur inverter. Of note, several genes reported down-regulated upon iron starvation in *S. aureus* (81) are IsrR targets predicted by Copra RNA, including *narG* and *nasD*.

In the absence of oxygen, Staphylococci use nitrate and nitrite as electron acceptors of the anaerobic respiratory chain (82). These products are provided to humans by

nutrients, and are also generated by oxidation of endogenous NO synthase products (83,84). Nitrate and nitrite reductases are expressed when needed, *i.e.*, in anaerobic conditions when nitrate is present. Their expression is controlled by the staphylococcal transcriptional regulator NreABC (85-87). *IsrR* provides an additional checkpoint to nitrate respiration by linking transcription of *narGHJ* and *nirR nasDEF* operons to the presence of iron, an essential element of these encoded respiratory chain components (Figure 8).



**Figure 8. *IsrR* control of dissimilatory nitrate reduction.**

*isrR* is Fur-regulated. Its expression is induced in iron-free growth conditions. *IsrR* base pairs to SDs of mRNAs encoding components of formate dehydrogenase, nitrate reductase, nitrite reductase and glutamate synthase, thus preventing translation of the encoded iron-sulfur containing enzymes and nitrate dissimilatory reduction.

When iron is limiting, *IsrR* targets various mRNAs expressing iron-containing proteins that are not essential for growth. The dissimilatory nitrate reduction pathway is inactivated, while *S. aureus* can still grow by fermentation in anaerobic conditions. sRNAs that directly affect nitrogen metabolism have been characterized in Alphaproteobacteria, Gammaproteobacteria and Cyanobacteria (88-90); in several cases, the sRNAs directly downregulate regulators of nitrogen respiratory pathways. To our knowledge, *IsrR* is the first sRNA example from the Firmicutes phylum. Biocomputing analysis lead us to speculate that *IsrR* could also regulate a regulator by targeting *nreC* mRNA (Supplementary Figure S5).

A characteristic feature of sRNAs in Firmicutes and in particular in *S. aureus* is the presence of one or several exposed CRRs that can act on the G-rich region like those of the Shine Dalgarno sequences. The interaction models proposed from the experiments probing nucleotide reactivity indicate the requirement of CRR2 for IsrR activity against *fdhA*, *gltB2* and *nasD* mRNAs. Translational activity assays with a reporter fusion system confirmed the CRR2 involvement. SHAPE footprinting experiments only detect state-changing nucleotides and not those involved in base pairing both in intramolecular and intermolecular contexts. From this, we propose that predicted intermolecular base pairs nucleate the complex, and could then be extended essentially as proposed by IntaRNA and include the other two CRRs of IsrR, creating a complex that efficiently inhibits mRNA translation despite their individual internal structures. IsrR pairing to *fdhA* and *gltB2* mRNAs acts primarily on translation rather than on mRNA stability, which could be probably the case also for *nasD* and *narG* mRNAs.

IsrR-spared iron can be reallocated to vital processes. Consequently, IsrR plays a central role for *S. aureus* adaptation, including resistance to host nutritional immunity. Preventing IsrR activity by dedicated RNA antisense molecules or other means would antagonize staphylococcal pathogenicity.

Most staphylococcal sRNAs are poorly conserved across different species (46). One explanation is that the *trans*-acting sRNAs mostly act by imperfect pairing to untranslated regions (*i.e.*, sRNAs and targeted UTRs); unlike ORFs, these sequences are prone to silent mutations and thus subject to rapid evolutionary changes. However, *isrR* and its Fur regulation are conserved throughout the *Staphylococcus* genus. This less common interspecies conservation (another example in *S. aureus* is RsaE (91)) reveals a selective pressure to maintain IsrR sequence, structure and regulation intact. IsrR pairing with several mRNA targets relate to important functions, such as iron homeostasis, which may explain this conservation; the occurrence of random *isrR* mutations would affect iron-related metabolism, and therefore be counter-selected.

sRNAs that down-regulate mRNAs encoding iron-sulfur clusters are found in Gram-positive and Gram-negative bacteria. In addition to IsrR, these include RyhB in enteric bacteria (5), the paralogs PrrF1 and PrrF2 in *P. aeruginosa* (11), MrsI in *M. tuberculosis* (15) and FsrA sRNA in *B. subtilis* (92). Remarkably, none of these sRNAs share the same

RNA chaperone dependency (*e.g.*, Hfq for RyhB and PrrF1/PrrF2; FbpA, FbpB and FbpC for FsrA) with IsrR, and their genes share neither synteny nor sequence homology. FsrA, but not the other sRNAs, binds to the Shine-Dalgarno sequence via a C-rich motif (92), but requires three chaperone functions. These sRNAs are therefore not IsrR orthologs. Nevertheless, they do share several target mRNAs encoding the same iron-sulfur-containing enzymes (Supplementary Table S7). Indeed, the iron-sparing response of these sRNAs includes targets involved in nitrate metabolism and the TCA cycle. The TCA cycle mRNA encoding aconitase (*acnA/citB*) is targeted by all the sRNAs discussed above. The common regulation of these sRNAs by iron and their shared targets suggest convergent evolution, which can be reasonably explained as follows: The accumulation of non-essential iron-containing enzymes is deleterious in iron-scarce environments; however, an sRNA induced during iron starvation that mutates to pair with these transcripts provides an immediate selective advantage, more energetically efficient than the production of specialized regulatory proteins. Since Fur is a widely conserved iron-dependent repressor in bacteria, any Fur-regulated sRNAs, possibly originating from spurious transcriptions, can be recruited to fulfill this task. RNAs responsive to Fe (rrF) are indeed common in bacteria (5,89). The long-term evolution of some rrFs is expected to lead *in fine* to the birth of IsrR/RyhB functional analogs.



## **DATA AVAILABILITY**

Data generated or analyzed during this study are presented. Strains and plasmids are available from the corresponding author on request.

## **FUNDING**

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## **AUTHOR CONTRIBUTIONS**

P.B. conceived the project. R.H.C.T., W. L., M. B. and P.B. designed experiments and interpreted the data. V.B. and B.F. designed, performed the animal experiments and interpreted the data. M.P., C.V. and B.S. designed, performed experiments and

interpreted the data to determine RNA structures and interactions. R.H.C.T. and P.B. wrote the manuscript. R.H.C.T., B.F., M.P., B.S. and P.B. edited the paper.

#### **COMPETING INTERESTS**

The authors declare no competing interests.

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## SUPPLEMENTARY DATA

### **srRNA-controlled iron sparing response in Staphylococci**

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**Table S1. *Staphylococcus aureus* strains**

**Table S2. Plasmids**

**Table S3. Primers**

**Table S4. Fitness library composition**

**Table S5. *IsrR* sequences in *Staphylococcus* genus**

**Table S6. Proteins containing an Fe-S cluster in *S. aureus***

**Table S7. *IsrR* functional analogs and their targets**

**Figure S1. Absence of *IsrR* is detrimental when iron is scarce**

**Figure S2. *isrR* complementation restores optimal growth in low-iron conditions**

**Figure S3. *IsrR* 5'/3'RACE mapping and LocARNA secondary structure prediction**

**Figure S4. *IsrR* secondary structure obtained with IPANEMAP and reactivity profile**

**Figure S5. *IsrR* putative targets**

**Figure S6. *IsrR* putative targets involved in nitrate respiration pathway**

**Figure S7. Electrophoretic mobility shift assay of *IsrR* in the presence of *fdhA* mRNA**

**Figure S8. Comparison of *nasD* and *narG* mRNA reactivity obtained in presence or  
absence of *IsrR* with proposed alternative interaction model**

**Figure S9. Reporter fusions associated to nitrate respiration for *IsrR* activity tests**

**Figure S10. Translational down-regulation by *IsrR* and CRR contribution**

**Figure S11. Hfq is not required for *IsrR* activity**

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**Table S1. Staphylococcus aureus strains**

Name	Relevant genotype	Reference or construction
RN4220	NCTC8325 derivative used for transformations with plasmids constructed in <i>E. coli pcnB</i> strain	(1)
8325-4	NCTC8325 derivative	(2)
HG003	NCTC8325 <i>rsbU</i> and <i>tcaR</i> repaired	(3)
<b><math>\Delta</math>sRNA tagged mutants for libraries (Figure 1B)</b>		
SAPhB618	as HG003 $\Delta$ rnalll::tag004	(4)
SAPhB347	as HG003 $\Delta$ rsaOG::tag009	(4)
SAPhB349	as HG003 $\Delta$ rsaG::tag011	(4)
SAPhB368	as HG003 $\Delta$ teg147::tag018	(4)
SAPhB380	as HG003 $\Delta$ rsaB::tag025	(4)
SAPhB682	as HG003 $\Delta$ rsaD::tag026	(4)
SAPhB386	as HG003 $\Delta$ teg116::tag030	(4)
SAPhB397	as HG003 $\Delta$ sau85::tag038	(4)
SAPhB402	as HG003 $\Delta$ sau6353::tag042	(4)
SAPhB404	as HG003 $\Delta$ rsaE::tag045	(4)
SAPhB412	as HG003 $\Delta$ ssr42::tag050	(4)
SAPhB415	as HG003 $\Delta$ teg155::tag053	(4)
SAPhB960	as HG003 $\Delta$ sprF3::tag070	HG003 + pIM- <i>sprF3</i> ::tag070
SAPhB961	as HG003 $\Delta$ sprF3::tag070	HG003 + pIM- <i>sprF3</i> ::tag070
SAPhB962	as HG003 $\Delta$ sprF3::tag070	HG003 + pIM- <i>sprF3</i> ::tag070
SAPhB862	as HG003 $\Delta$ sRNA334::tag073	HG003 + pIM- <i>sRNA334</i> ::tag073
SAPhB863	as HG003 $\Delta$ sRNA334::tag073	HG003 + pIM- <i>sRNA334</i> ::tag073
SAPhB864	as HG003 $\Delta$ sRNA334::tag073	HG003 + pIM- <i>sRNA334</i> ::tag073
SAPhB943	as HG003 $\Delta$ rsaA::tag075	HG003 + pIM- <i>rsaA</i> ::tag075
SAPhB944	as HG003 $\Delta$ rsaA::tag075	HG003 + pIM- <i>rsaA</i> ::tag075
SAPhB945	as HG003 $\Delta$ rsaA::tag075	HG003 + pIM- <i>rsaA</i> ::tag075
SAPhB890	as HG003 $\Delta$ sau76::tag076	HG003 + pIM- <i>sau76</i> ::tag076
SAPhB891	as HG003 $\Delta$ sau76::tag076	HG003 + pIM- <i>sau76</i> ::tag076
SAPhB962	as HG003 $\Delta$ sau76::tag076	HG003 + pIM- <i>sau76</i> ::tag076
SAPhB883	as HG003 $\Delta$ rsaOl::tag077	HG003 + pIM- <i>rsaOl</i> ::tag077
SAPhB884	as HG003 $\Delta$ rsaOl::tag077	HG003 + pIM- <i>rsaOl</i> ::tag077
SAPhB885	as HG003 $\Delta$ rsaOl::tag077	HG003 + pIM- <i>rsaOl</i> ::tag077
SAPhB865	as HG003 $\Delta$ teg16::tag080	HG003 + pIM- <i>teg16</i> ::tag080
SAPhB866	as HG003 $\Delta$ teg16::tag080	HG003 + pIM- <i>teg16</i> ::tag080
SAPhB867	as HG003 $\Delta$ teg16::tag080	HG003 + pIM- <i>teg16</i> ::tag080
SAPhB871	as HG003 $\Delta$ sRNA287::tag085	HG003 + pIM- <i>sRNA287</i> ::tag085
SAPhB872	as HG003 $\Delta$ sRNA287::tag085	HG003 + pIM- <i>sRNA287</i> ::tag085
SAPhB873	as HG003 $\Delta$ sRNA287::tag085	HG003 + pIM- <i>sRNA287</i> ::tag085
SAPhB874	as HG003 $\Delta$ sRNA71::tag086	HG003 + pIM- <i>sRNA71</i> ::tag086
SAPhB875	as HG003 $\Delta$ sRNA71::tag086	HG003 + pIM- <i>sRNA71</i> ::tag086
SAPhB876	as HG003 $\Delta$ sRNA71::tag086	HG003 + pIM- <i>sRNA71</i> ::tag086
SAPhB907	as HG003 $\Delta$ sRNA209::tag093	HG003 + pIM- <i>sRNA209</i> ::tag093

SAPhB908	as HG003 $\Delta$ sRNA209::tag093	HG003 + pIM-sRNA209::tag093
SAPhB909	as HG003 $\Delta$ sRNA209::tag093	HG003 + pIM-sRNA209::tag093
SAPhB899	as HG003 $\Delta$ teg106::tag095	HG003 + pIM-teg106::tag095
SAPhB900	as HG003 $\Delta$ teg106::tag095	HG003 + pIM-teg106::tag095
SAPhB946	as HG003 $\Delta$ teg106::tag095	HG003 + pIM-teg106::tag095
SAPhB921	as HG003 $\Delta$ sRNA260::tag096	HG003 + pIM-sRNA260::tag096
SAPhB922	as HG003 $\Delta$ sRNA260::tag096	HG003 + pIM-sRNA260::tag096
SAPhB947	as HG003 $\Delta$ sRNA260::tag096	HG003 + pIM-sRNA260::tag096
SAPhB910	as HG003 $\Delta$ sRNA345::tag097	HG003 + pIM-sRNA345::tag097
SAPhB911	as HG003 $\Delta$ sRNA345::tag097	HG003 + pIM-sRNA345::tag097
SAPhB912	as HG003 $\Delta$ sRNA345::tag097	HG003 + pIM-sRNA345::tag097
SAPhB932	as HG003 $\Delta$ ncRNA2::tag099	HG003 + pIM-ncRNA2::tag099
SAPhB933	as HG003 $\Delta$ ncRNA2::tag099	HG003 + pIM-ncRNA2::tag099
SAPhB934	as HG003 $\Delta$ ncRNA2::tag099	HG003 + pIM-ncRNA2::tag099
SAPhB940	as HG003 $\Delta$ ncRNA3::tag100	HG003 + pIM-ncRNA3::tag100
SAPhB941	as HG003 $\Delta$ ncRNA3::tag100	HG003 + pIM-ncRNA3::tag100
SAPhB942	as HG003 $\Delta$ ncRNA3::tag100	HG003 + pIM-ncRNA3::tag100
SAPhB954	as HG003 $\Delta$ ssrS::tag107	HG003 + pIM-ssrS::tag107
SAPhB955	as HG003 $\Delta$ ssrS::tag107	HG003 + pIM-ssrS::tag107
SAPhB956	as HG003 $\Delta$ ssrS::tag107	HG003 + pIM-ssrS::tag107
SAPhB1006	as HG003 $\Delta$ sprF1::tag110	HG003 + pIM-sprF1::tag110
SAPhB1007	as HG003 $\Delta$ sprF1::tag110	HG003 + pIM-sprF1::tag110
SAPhB1008	as HG003 $\Delta$ sprF1::tag110	HG003 + pIM-sprF1::tag110
SAPhB974	as HG003 $\Delta$ sprX2::tag111	HG003 + pIM-sprX2::tag111
SAPhB975	as HG003 $\Delta$ sprX2::tag111	HG003 + pIM-sprX2::tag111
SAPhB997	as HG003 $\Delta$ sprX2::tag111	HG003 + pIM-sprX2::tag111
SAPhB978	as HG003 $\Delta$ sprY2::tag112	HG003 + pIM-sprY2::tag112
SAPhB979	as HG003 $\Delta$ sprY2::tag112	HG003 + pIM-sprY2::tag112
SAPhB980	as HG003 $\Delta$ sprY2::tag112	HG003 + pIM-sprY2::tag112
SAPhB957	as HG003 $\Delta$ sprY3::tag113	HG003 + pIM-sprY3::tag113
SAPhB958	as HG003 $\Delta$ sprY3::tag113	HG003 + pIM-sprY3::tag113
SAPhB959	as HG003 $\Delta$ sprY3::tag113	HG003 + pIM-sprY3::tag113
SAPhB901	as HG003 $\Delta$ sau41::Tag115	HG003 + pIM-sau41::Tag115
SAPhB902	as HG003 $\Delta$ sau41::Tag115	HG003 + pIM-sau41::Tag115
SAPhB903	as HG003 $\Delta$ sau41::Tag115	HG003 + pIM-sau41::Tag115
SAPhB948	as HG003 $\Delta$ sau5949::tag117	HG003 + pIM-sau5949::tag117
SAPhB949	as HG003 $\Delta$ sau5949::tag117	HG003 + pIM-sau5949::tag117
SAPhB950	as HG003 $\Delta$ sau5949::tag117	HG003 + pIM-sau5949::tag117
SAPhB966	as HG003 $\Delta$ sprF2::tag118	HG003 + pIM-sprF2::tag118
SAPhB967	as HG003 $\Delta$ sprF2::tag118	HG003 + pIM-sprF2::tag118
SAPhB998	as HG003 $\Delta$ sprF2::tag118	HG003 + pIM-sprF2::tag118
SAPhB1031	as HG003 $\Delta$ sprB::tag121	HG003 + pIM-sprB::tag121
SAPhB1032	as HG003 $\Delta$ sprB::tag121	HG003 + pIM-sprB::tag121
SAPhB1033	as HG003 $\Delta$ sprB::tag121	HG003 + pIM-sprB::tag121
SAPhB1242	as HG003 $\Delta$ rsaC::tag133	HG003 + pIM-rsaC::tag133

SAPhB1243	as HG003 $\Delta$ rsaC::tag133	HG003 + pIM-rsaC::tag133
SAPhB1244	as HG003 $\Delta$ rsaC::tag133	HG003 + pIM-rsaC::tag133
SAPhB1234	as HG003 $\Delta$ S204::tag134	HG003 + pIM-S204::tag134
SAPhB1235	as HG003 $\Delta$ S204::tag134	HG003 + pIM-S204::tag134
SAPhB1236	as HG003 $\Delta$ S204::tag134	HG003 + pIM-S204::tag134
SAPhB1231	as HG003 $\Delta$ isrR::tag135	HG003 + pIM-S596::tag135
SAPhB1232	as HG003 $\Delta$ isrR::tag135	HG003 + pIM-S596::tag135
SAPhB1233	as HG003 $\Delta$ isrR::tag135	HG003 + pIM-S596::tag135
SAPhB1239	as HG003 $\Delta$ S808::tag137	HG003 + pIM-S808::tag137
SAPhB1240	as HG003 $\Delta$ S808::tag137	HG003 + pIM-S808::tag137
SAPhB1241	as HG003 $\Delta$ S808::tag137	HG003 + pIM-S808::tag137
SAPhB1015	locus3::tag139	HG003 + pIM-locus3::tag139
SAPhB1016	locus3::tag139	HG003 + pIM-locus3::tag139
SAPhB1017	locus3::tag139	HG003 + pIM-locus3::tag139
SAPhB1012	locus2::tag140	HG003 + pIM-locus2::tag140
SAPhB1013	locus2::tag140	HG003 + pIM-locus2::tag140
SAPhB1014	locus2::tag140	HG003 + pIM-locus2::tag140
SAPhB1009	locus1::tag141	HG003 + pIM-locus1::tag141
SAPhB1010	locus1::tag141	HG003 + pIM-locus1::tag141
SAPhB1011	locus1::tag141	HG003 + pIM-locus1::tag141
SAPhB1018	as HG003 $\Delta$ sau5971::tag142	HG003 + pIM-sau5971::tag142
SAPhB1019	as HG003 $\Delta$ sau5971::tag142	HG003 + pIM-sau5971::tag142
SAPhB1020	as HG003 $\Delta$ sau5971::tag142	HG003 + pIM-sau5971::tag142
SAPhB976	as HG003 $\Delta$ sprA1::tag144	HG003 + pIM-sprA1::tag144
SAPhB977	as HG003 $\Delta$ sprA1::tag144	HG003 + pIM-sprA1::tag144
SAPhB996	as HG003 $\Delta$ sprA1::tag144	HG003 + pIM-sprA1::tag144
SAPhB1027	as HG003 $\Delta$ sprX2::tag145 $\Delta$ sprX1::tag149	SAPhB976 + pIM-sprX2::tag145
SAPhB1028	as HG003 $\Delta$ sprX2::tag145 $\Delta$ sprX1::tag149	SAPhB976 + pIM-sprX2::tag145
SAPhB1029	as HG003 $\Delta$ sprX2::tag145 $\Delta$ sprX1::tag149	SAPhB976 + pIM-sprX2::tag145
SAPhB1003	as HG003 $\Delta$ sprX1::tag146	HG003 + pIM-sprX1::tag146
SAPhB1004	as HG003 $\Delta$ sprX1::tag146	HG003 + pIM-sprX1::tag146
SAPhB1005	as HG003 $\Delta$ sprX1::tag146	HG003 + pIM-sprX1::tag146
SAPhB971	as HG003 $\Delta$ rsaH::tag147	HG003 + pIM-rsaH::tag147
SAPhB972	as HG003 $\Delta$ rsaH::tag147	HG003 + pIM-rsaH::tag147
SAPhB973	as HG003 $\Delta$ rsaH::tag147	HG003 + pIM-rsaH::tag147
SAPhB1021	as HG003 $\Delta$ sprY1::tag148	HG003 + pIM-sprY1::tag148
SAPhB1022	as HG003 $\Delta$ sprY1::tag148	HG003 + pIM-sprY1::tag148
SAPhB1023	as HG003 $\Delta$ sprY1::tag148	HG003 + pIM-sprY1::tag148
<b>IsrR complementation studies (Figure 1C and S2)</b>		
SAPhB1372	as HG003 $\Delta$ isrR::tag135 pCN38	SAPhB1231 + pCN38
SAPhB1373	as HG003 $\Delta$ isrR::tag135 pCN38-IsrR	SAPhB1231 + pCN38-IsrR
SAPhB1500	as HG003 $\Delta$ isrR::tag135 locus2::isrR <sup>+</sup>	SAPhB1231 + pIM-locus2::isrR <sup>+</sup>
SAPhB1502	as HG003 $\Delta$ isrR::tag135 locus3::isrR <sup>+</sup>	SAPhB1231 + pIM-locus3::isrR <sup>+</sup>
<b>Fur regulation (Figure 2)</b>		
MJH010	as NCTC8325-4 $\Delta$ fur::tetR	(5)

SAPhB1542	as HG003 $\Delta fur::tetR$	HG003 + $\phi 80$ on MJH010
SAPhB1558	as NCTC8325-4 pP <sub>IsrR</sub>	NCTC8325-4 + pP <sub>IsrR</sub>
SAPhB1550	as NCTC8325-4 pP <sub>IsrR</sub> :: <i>gfp</i>	NCTC8325-4 + pP <sub>IsrR</sub> :: <i>gfp</i>
SAPhB1552	as NCTC8325-4 pP <sub>IsrR1</sub> :: <i>gfp</i>	NCTC8325-4 + pP <sub>IsrR1</sub> :: <i>gfp</i>
SAPhB1554	as NCTC8325-4 pP <sub>IsrR2</sub> :: <i>gfp</i>	NCTC8325-4 + pP <sub>IsrR2</sub> :: <i>gfp</i>
SAPhB1556	as NCTC8325-4 pP <sub>IsrR1&amp;2</sub> :: <i>gfp</i>	NCTC8325-4 + pP <sub>IsrR1&amp;2</sub> :: <i>gfp</i>
<b>pRMC2-IsrR derivatives</b>		
SAPhB1801	as HG003 pRMC2 $\Delta$ R	HG003 + pRMC2 $\Delta$ R
SAPhB1517	as HG003 pRMC2 $\Delta$ R-IsrR	HG003 + pRMC2 $\Delta$ R-IsrR
SAPhB1618	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R	SAPhB1231 + pRMC2 $\Delta$ R
SAPhB1519	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR	SAPhB1231 + pRMC2 $\Delta$ R-IsrR
SAPhB1568	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR1	SAPhB1231 + pRMC2 $\Delta$ R-IsrR $\Delta$ CRR1
SAPhB1674	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR2	SAPhB1231 + pRMC2 $\Delta$ R-IsrR $\Delta$ CRR2
SAPhB1703	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR3	SAPhB1231 + pRMC2 $\Delta$ R-IsrR $\Delta$ CRR3
<b>IsrR/<i>gltB2</i> mRNA pairing reporter assay (Figure 6)</b>		
SAPhB1628	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R p5' <i>GltB2</i> -GFP	SAPhB1618 + p5' <i>GltB2</i> -GFP
SAPhB1598	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR p5' <i>GltB2</i> -GFP	SAPhB1519 + p5' <i>GltB2</i> -GFP
SAPhB1600	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR1 p5' <i>GltB2</i> -GFP	SAPhB1568 + p5' <i>GltB2</i> -GFP
SAPhB1717	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR2 p5' <i>GltB2</i> -GFP	SAPhB1674 + p5' <i>GltB2</i> -GFP
SAPhB1721	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR3 p5' <i>GltB2</i> -GFP	SAPhB1703 + p5' <i>GltB2</i> -GFP
<b>IsrR/<i>fdhA</i> mRNA pairing reporter assay (Figure 6)</b>		
SAPhB1745	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R p5' <i>FdhA</i> -GFP	SAPhB1618 + p5' <i>FdhA</i> -GFP
SAPhB1747	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR p5' <i>FdhA</i> -GFP	SAPhB1519 + p5' <i>FdhA</i> -GFP
SAPhB1749	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR1 p5' <i>FdhA</i> -GFP	SAPhB1568 + p5' <i>FdhA</i> -GFP
SAPhB1751	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR2 p5' <i>FdhA</i> -GFP	SAPhB1674 + p5' <i>FdhA</i> -GFP
SAPhB1753	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR3 p5' <i>FdhA</i> -GFP	SAPhB1703 + p5' <i>FdhA</i> -GFP
<b>IsrR/<i>narG</i> mRNA pairing reporter assay (Figure S10)</b>		
SAPhB1624	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R p5' <i>NarG</i> -GFP	SAPhB1618 + p5' <i>NarG</i> -GFP
SAPhB1590	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR p5' <i>NarG</i> -GFP	SAPhB1519 + p5' <i>NarG</i> -GFP
SAPhB1592	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR1 p5' <i>NarG</i> -GFP	SAPhB1568 + p5' <i>NarG</i> -GFP
SAPhB1741	as HG003 $\Delta isrR::tag135$ pRMC2 $\Delta$ R-IsrR $\Delta$ CRR2 p5' <i>NarG</i> -GFP	SAPhB1674 + p5' <i>NarG</i> -GFP

SAPhB1743	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R- IsrR $\Delta$ CRR3 p5'NarG-GFP	SAPhB1703 + p5'NarG-GFP
<b>IsrR/nasD mRNA pairing reporter assay (Figure S10)</b>		
SAPhB1626	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R p5'NasD-GFP	SAPhB1618 + p5'NasD-GFP
SAPhB1594	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R-IsrR p5'NasD-GFP	SAPhB1519 + p5'NasD-GFP
SAPhB1596	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R- IsrR $\Delta$ CRR1 p5'NasD-GFP	SAPhB1568 + p5'NasD-GFP
SAPhB1781	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R- IsrR $\Delta$ CRR2 p5'NasD-GFP	SAPhB1674 + p5'NasD-GFP
SAPhB1783	as HG003 $\Delta$ <i>isrR</i> ::tag135 pRMC2 $\Delta$ R- IsrR $\Delta$ CRR3 p5'NasD-GFP	SAPhB1703 + p5'NasD-GFP
<b>hfq derivatives (Figure S11)</b>		
SAPhB1024	as HG003 $\Delta$ <i>hfq</i> ::tag143	(4)
SAPhB1709	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R	SAPhB1024 + pRMC2 $\Delta$ R
SAPhB1711	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R-IsrR	SAPhB1024 + pRMC2 $\Delta$ R-IsrR
SAPhB1807	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R p5'FdhA- GFP	SAPhB1709+ p5'FdhA-GFP
SAPhB1809	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R-IsrR p5'FdhA-GFP	SAPhB1711+ p5'FdhA-GFP
SAPhB1811	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R p5'NarG- GFP	SAPhB1709 + p5'NarG-GFP
SAPhB1813	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R-IsrR p5'NarG-GFP	SAPhB1711 + p5'NarG-GFP
SAPhB1815	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R p5'NasD- GFP	SAPhB1709 + p5'NasD-GFP
SAPhB1817	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R-IsrR p5'NasD-GFP	SAPhB1711 + p5'NasD-GFP
SAPhB1819	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R p5'GltB2-GFP	SAPhB1709 + p5'GltB2-GFP
SAPhB1821	as HG003 $\Delta$ <i>hfq</i> ::tag143 pRMC2 $\Delta$ R-IsrR p5'GltB2-GFP	SAPhB1711 + p5'GltB2-GFP
SAPhB2056	as HG003 p5'FdhA-GFP	HG003 + p5'FdhA-GFP
SAPhB2057	as HG003 $\Delta$ <i>isrR</i> ::tag135 p5'FdhA-GFP	SAPhB1231 + p5'FdhA-GFP
SAPhB2058	as HG003 $\Delta$ <i>hfq</i> ::tag143 p5'FdhA-GFP	SAPhB1024 + p5'FdhA-GFP

**Table S2. Plasmids**

Name	Relevant properties	Reference /Construction*
<b>Mutant library constructions</b>		
pIMAY	Shuttle <i>rep</i> (Ts) vector in <i>S. aureus</i>	(6)
pIM- <i>sprF3</i> ::tag070	<i>sprF3</i> replacement by tag070	up: 2147/2148; dw: 2149/2150
pIM- <i>sRNA334</i> ::tag07	<i>sRNA334</i> replacement by tag073	up:1959/1960; dw; 1961/1962
pIM- <i>rsaA</i> ::tag075	<i>rsaA</i> replacement by tag075	up: 1928/1929; dw: 1930/1931
pIM- <i>sau76</i> ::tag076	<i>sau76</i> replacement by tag076	up: 1836/1837; dw: 1838/1839
pIM- <i>rsaOI</i> ::tag077	<i>rsaOI</i> replacement by tag077	up:1840/1841; dw: 1842/1843
pIM- <i>teg16</i> ::tag080	<i>teg16</i> replacement by tag080	up: 1931/1932; dw: 1933/1934
pIM- <i>sRNA287</i> ::tag085	<i>sRNA287</i> replacement by tag085	up: 1952/1953; dw: 1954/1955
pIM- <i>sRNA71</i> ::tag086	<i>sRNA71</i> replacement by tag086	up: 1910/1911; dw: 1912/1913
pIM- <i>sRNA209</i> ::tag093	<i>sRNA209</i> replacement by tag093	up:1987/1988; dw: 1989/1990
pIM- <i>teg106</i> ::tag095	<i>teg106</i> replacement by tag095	up2001/2002; dw: 2003/2004
pIM- <i>sRNA260</i> ::tag096	<i>sRNA260</i> replacement by t	up: 2008/2009; dw: 2009/2010
pIM- <i>sRNA345</i> ::tag097	<i>sRNA345</i> replacement by tag097	up: 2015/2016; dw: 2017/2018
pIM- <i>ncRNA2</i> ::tag099	<i>ncRNA2</i> replacement by tag099	up: 2031/2032; dw: 2033/2034
pIM- <i>ncRNA3</i> ::tag100	<i>ncRNA3</i> replacement by tag100	up: 2038/2039; dw: 2040/2041
pIM- <i>ssrS</i> ::tag107	<i>ssrS</i> replacement by tag107	up: 2019/2020; dw: 2021/2022
pIM- <i>sprF1</i> ::tag110	<i>sprF1</i> replacement by tag110	up: 2161/2162; dw: 2163/2164
pIM- <i>sprX2</i> ::tag111	<i>sprX2</i> replacement by tag111	up: 2189/2190; dw: 2191/2192
pIM- <i>sprY2</i> ::tag112	<i>sprY2</i> replacement by tag112	up: 2196/2197; dw: 2198/2199
pIM- <i>sprY3</i> ::tag113	<i>sprY3</i> replacement by tag113	up: 2168/2169; dw: 2170/2171
pIM- <i>sau41</i> ::Tag115	<i>sau41</i> replacement by Tag115	up:1966/1967; dw; 1968/1969
pIM- <i>sau5949</i> ::tag117	<i>sau5949</i> replacement by tag117	up: 2066/2067; dw: 2068/2069
pIM- <i>sprF2</i> ::tag118	<i>sprF2</i> replacement by tag118	up: 2026/2027; dw: 2028/2029
pIM- <i>rsaC</i> ::tag133	<i>rsaC</i> replacement by tag133	up: 2395/2396; dw: 2397/2398



pIM-S204::tag134	S204 replacement by tag134	up: 2387/2388; dw: 2389/2390
pIM-S596::tag135	S596 replacement by tag135	up: 2379/2380; dw: 2381/2382
pIM-S808::tag137	S808 replacement by tag137	up: 2363/2364; dw: 2365/2366
pIM-locus3::tag139	locus3 replacement by tag139	up: 2234/2235; dw: 2236/2237
pIM-locus2::tag140	locus2 replacement by tag140	up: 2222/2223; dw: 2224/2225
pIM-locus1::tag141	locus1 replacement by tag141	up: 2228/2229; dw: 2230/2231
pIM-sau5971::tag142	sau5971 replacement by tag142	up: 2215/2216; dw: 2217/2218
pIM-sprA1::tag144	sprA1 replacement by tag144	up: 2140/2141; dw: 2142/2143
pIM-sprX2::tag145	sprX2 replacement by tag145	up: 2189/2190; dw: 2191/2192
pIM-sprX1::tag149	sprX1 replacement by tag149	up: 2175/2176; dw: 2177/2178
pIM-sprX1::tag146	sprX1 replacement by tag146	up: 2175/2176; dw: 2177/2178
pIM-rsaH::tag147	rsaH replacement by tag147	up: 2201/2202; dw: 2203/2204
pIM-sprY1::tag148	sprY1 replacement by tag148	up: 2182/2183; dw: 2184/2185
<b>isrR complementation and toxicity</b>		
pCN38	Shuttle vector, pT181 replicon, Cm <sup>R</sup>	(7)
pCN38-IsrR	<i>isrR</i> under the control of its endogenous promoter	1489/1490 on pCN38 + 2343/2344 on HG003
pIM-locus2	SAOUHSC_03030-locus2-SAOUHSC_03031 region	1536/1537 on pIMAY + 2228/2231 on HG003
pIM-locus2::isrR <sup>+</sup>	For <i>isrR</i> insertion at HG003 locus2	2507/2508 on pIM-locus2 + 2511/2512 on HG003
pIM-locus3	SAOUHSC_01263-locus3-SAOUHSC_01264 region	1536/1537 + pIM-locus3 + 2234/2237 on HG003
pIM-locus3::isrR <sup>+</sup>	For <i>isrR</i> insertion at HG003 locus3	2509/2510 pIM-locus3 + 2511/2512 HG003
<b>Fur-dependent isrR regulation</b>		
pP <sub>isrR</sub> ::GFP	Translational fusion between <i>isrR</i> promoter region and <i>sgfp</i>	2546/ 2547 on pCN38-isrR + 2548/2549 on pCM11
pP <sub>isrR1</sub> ::GFP	pP <sub>isrR</sub> ::GFP with the <i>isrR</i> Fur motif 1 mutated.	2573/2574 on pP <sub>isrR</sub> ::GFP
pP <sub>isrR2</sub> ::GFP	pP <sub>isrR</sub> ::GFP with the <i>isrR</i> Fur motif 2 mutated.	2575/2576 on pP <sub>isrR</sub> ::GFP
pP <sub>isrR1&amp;2</sub> ::GFP	pP <sub>isrR</sub> ::GFP with the <i>isrR</i> Fur motif 1 & 2 mutated.	2575/2576 on pP <sub>isrR1</sub> ::GFP
<b>Constitutive expression of isrR and mutated derivatives</b>		



pRMC2	Anhydrotetracycline (aTc) inducible promoter	(8)
pRMC2ΔR	Deletion of <i>tetR</i> from pRMC2	2538/2539 on pRMC2
pRMC2-IsrR	Inducible expression of <i>IsrR</i>	856/918 on pRMC2 with + PCR 2499/2500 on HG003 with
pRMC2ΔR-IsrR	Deletion of <i>tetR</i> from pRMC2-IsrR: Constitutive expression of <i>IsrR</i>	2538/2539 on pRMC2-IsrR
pRMC2ΔR-IsrRΔCRR1	Constitutive expression of <i>IsrR</i> ΔCRR1	2605/2606 on pRMC2ΔR-IsrR
pRMC2ΔR-IsrRΔCRR2	Constitutive expression of <i>IsrR</i> ΔCRR2	2631/2632 on pRMC2ΔR-IsrR
pRMC2ΔR-IsrRΔCRR3	Constitutive expression of <i>IsrR</i> ΔCRR3	2667/2668 on pRMC2ΔR-IsrR
<b>Targets 5'UTR in translational fusion with <i>gfp</i></b>		
pCN34	Shuttle vector, pT181 replicon, Km <sup>R</sup>	(7)
pCM11	Promoter-less <i>sgfp</i> transcriptional reporter	(9)
pECTO	pMAD2 derivative to integrate DNA sequences between SAOUHSC_00278 and SAOUHSC_00279	Laboratory collection <a href="https://www.addgene.org/">https://www.addgene.org/</a>
pCN34- <i>gfp</i>	<i>rrnB</i> terminator from pECTO and <i>sarA</i> promoter, <i>sod</i> RBS and <i>sgfp</i> from pCM11	2554/2555 on pECTO + 2552/2553 on pCM11 + 2534/2535 on pCN34
p5'GltB2-GFP	Translational fusion between <i>gltB2</i> 5'UTR and <i>gfp</i> , under the control of P <sub>sarA</sub> (P <sub>sarA</sub> 5'UTR:: <i>gltB2</i> :: <i>gfp</i> )	2600/2601 on pCN34- <i>gfp</i> + 2621/2622 on HG003
p5'FdhA-GFP	Translational fusion between <i>fdhA</i> 5'UTR and <i>gfp</i> , under the control of P <sub>sarA</sub> (P <sub>sarA</sub> 5'UTR:: <i>fdhA</i> :: <i>gfp</i> )	2600/2601 on pCN34- <i>gfp</i> + 2694/2695 on HG003
p5'NarG-GFP	Translational fusion between <i>narG</i> 5'UTR and <i>gfp</i> , under the control of P <sub>sarA</sub> (P <sub>sarA</sub> 5'UTR:: <i>narG</i> :: <i>gfp</i> )	2600/2601 on pCN34- <i>gfp</i> + 2617/2618 on HG003
p5'NasD-GFP	Translational fusion between <i>nasD</i> 5'UTR and <i>gfp</i> , under the control of P <sub>sarA</sub> (P <sub>sarA</sub> 5'UTR:: <i>nasD</i> :: <i>gfp</i> )	2600/2601 on pCN34- <i>gfp</i> + 2619/2620 on HG003
<b><i>hfq</i> inactivation</b>		
pIM-hfq::tag143	<i>hfq</i> replacement by tag143	up: 2208/2209; dw: 2210/2211

\* #/# indicates primer couples used for PCR amplifications. For primer sequences, see Table S3. Plasmids were constructed by isothermal assembly of PCR product(s) (10). pIM plasmids: pIMAY derivatives for *S. aureus* chromosomal modifications were constructed by the assembly of PCR products from i) pIMAY (amplified with primers 1536 and 1537), ii) upstream and iii) downstream regions of each HG003 modified regions. Primers used for the amplifications these regions (about 0.8 to 1kb) are indicated, "up" and "dw" in the table, respectively. iv) Deleted genes were replaced by specific tag sequences amplified with primers 1870 and 1871 from a partially random primer, as described (4). For these, the assembly mixt contained an additional PCR product corresponding to amplified DNA tag sequences.

**Table S3. Primers**

Name	Description, PCR amplified region*	Sequence
856	pRMC2 F	GGTACCGTTAACAGATCTGAG
918	pRMC2 R	GCTTATTTTAATTATACTCTATCAATGATAGAG
1489	pCN38 amplification-F	CAGTTGCGCAGCCTGAATGG
1490	pCN38 amplification-R	CCTCTAGAGTCGACCTGCAG
1536	pIMAY F	GGTACCCAGCTTTTGTTCCTTTAGTGAGG
1537	pIMAY R	GAGCTCCAATTCGCCCTATAGTGAGTCG
1828	pIMAY-Up-RsaA-F	CGACTCACTATAGGGCGAATTGGAGCTCAGTTGCCAAGT CACCTGTTG
1829	pIMAY-Up-RsaA-R	GCGTATGGACCTAGGTATATCTCTATAACAATTTTTGTAA TGTTAACT
1830	pIMAY-Down-RsaA-F	ACCCACAACCTAGGTATATCTCGGGTACACTTTGCTAT GAG
1831	pIMAY-Down-RsaA-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGGATGCAC TTGTCCTGAA
1836	pIMAY-Up-sau76-F	CGACTCACTATAGGGCGAATTGGAGCTCGGTATCCTAGA CTACCTGCTAA
1837	pIMAY-Up-sau76-R	GCGTATGGACCTAGGTATATTAATGTATTATCAATAACA AAGTACA
1838	pIMAY-Down-sau76-F	ACCCACAACCTAGGTATATATAAACGAAAAATTTCCAAG CTTAAACC
1839	pIMAY-Down-sau76-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCAAACATGAG TCAAGCAGCCG
1840	pIMAY -Up-RsaOI-F	CGACTCACTATAGGGCGAATTGGAGCTCGCAACACAACC AGAAAGAGATAAC
1841	pIMAY -Up-RsaOI-R	GCGTATGGACCTAGGTATATCTTTATTACGGCTAATTAC AGTTCTCAA
1842	pIMAY -Down-RsaOI-F	ACCCACAACCTAGGTATATTACAGTATCAAATTTATCT AGGGC
1843	pIMAY -Down-RsaOI-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGCAGCTTA TCTCCACTGCT
1870	Tag amplification-R	GGTCTCTGAGATCCATACGCAGCTATGCAAT
1871	Tag amplification-F	GGTCTCATGTGTTGTGGGGTACAGCAATGAC
1910	pIMAY-Up-sRNA71-F	CGACTCACTATAGGGCGAATTGGAGCTCTGGTGGTAAGT CTGTTGAAAAGA
1911	pIMAY-Up-sRNA71-R	GCGTATGGACCTAGGTATATGTAACACATCACTTGATTA AAGACAATAC
1912	pIMAY-Down-sRNA71-F	ACCCACAACCTAGGTATATGTCAAATACTCGCTTTTTT ATTTCC
1913	pIMAY-Down-sRNA71-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCCATTTCC AAATTTGATGTGC
1931	pIMAY-Up-Teg16-F	CGACTCACTATAGGGCGAATTGGAGCTCTGAACCAGGAC CTTCAGCAA
1932	pIMAY-Up-Teg16-R	GCGTATGGACCTAGGTATATAATTTACTATATCTGCTTT AGTATGTCAAC
1933	pIMAY-Down-Teg16-F	ACCCACAACCTAGGTATATGTTTGAATGGGACTTGTA ACGT
1934	pIMAY-Down-Teg16-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGGAATCAT TTCTCGTCGCT

1952	pIMAY-Up-sRNA287-F	CGACTCACTATAGGGCGAATTGGAGCTCAATTCATGGCG GTTGTGGTG
1953	pIMAY-Up-sRNA287-R	GCGTATGGACCTAGGTATATCGATTAGGTCATGCAGATG T
1954	pIMAY-Down-sRNA287-F	ACCCACAACCTAGGTATATTATAGAAGCTGCGAACAGGT G
1955	pIMAY-Down-sRNA287-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCAGACACAGG CAAAATGAGTT
1959	pIMAY-Up-sRNA334-F	CGACTCACTATAGGGCGAATTGGAGCTCAGCAAATATCT CTTCTCCAACCA
1960	pIMAY-Up-sRNA334-R	GCGTATGGACCTAGGTATATTCTACTTTAAATTATCATC TCCATACTATT
1961	pIMAY-Down-sRNA334-F	ACCCACAACCTAGGTATATGTTAAACCTTAAACAAGA AATATTATTCA
1962	pIMAY-Down-sRNA334-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCAGCATGGTT TATCATTTGGCTCA
1966	pIMAY-Up-Sau41-F	CGACTCACTATAGGGCGAATTGGAGCTCTGCTAAAGATG CAAACGACGT
1967	pIMAY-Up-Sau41-R	GCGTATGGACCTAGGTATATTTTCATTGTACATAGTTATC TTGTGCGT
1968	pIMAY-Down-Sau41-F	ACCCACAACCTAGGTATATACTTAAAATTCTCAGGCCA CTATACC
1969	pIMAY-Down-Sau41-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTCTGAAGCG CAACAAACACA
1987	pIMAY-Up-sRNA209-F	CGACTCACTATAGGGCGAATTGGAGCTCAAACCCACACC GTTAGCAAC
1988	pIMAY-Up-sRNA209-R	GCGTATGGACCTAGGTATATTGACGCATCATACTATATT ACTGAAATTC
1989	pIMAY-Down-sRNA209-F	ACCCACAACCTAGGTATATAAATAACCACGTCCATCGA GA
1990	pIMAY-Down-sRNA209-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGCGTCATA ATTCCACAAGG
2001	pIMAY-Up-Teg106-F	CGACTCACTATAGGGCGAATTGGAGCTCTCTGGTAGGAC TATTGAATTTGCA
2002	pIMAY-Up-Teg106-R	GCGTATGGACCTAGGTATATCCATTCACCATATGATTTT TATTAATAGTT
2003	pIMAY-Down-Teg106-F	ACCCACAACCTAGGTATATCGTCTTGAAATGCTCCCTT CA
2004	pIMAY-Down-Teg106-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTCGCCATCT TCACCAAGTTC
2008	pIMAY-Up-sRNA260-F	CGACTCACTATAGGGCGAATTGGAGCTCAACGCAACCAA GTGATGTTG
2009	pIMAY-Up-sRNA260-R	GCGTATGGACCTAGGTATATCATAACAAAACCTCCTAATG TACTAGTTTAG
2010	pIMAY-Down-sRNA260-F	ACCCACAACCTAGGTATATGAACGTGCATCAGTCCTAA G
2011	pIMAY-Down-sRNA260-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCGTTTCGAGG ATTCACTGTTTCG
2015	pIMAY-Up-sRNA345-F	CGACTCACTATAGGGCGAATTGGAGCTCGTATTCTCTGA AGACGTTTGAACA
2016	pIMAY-Up-sRNA345-R	GCGTATGGACCTAGGTATATCTGTCTGACACCTTGATAT TAAAGGATTTTC
2017	pIMAY-Down-sRNA345-F	ACCCACAACCTAGGTATATCGTTTTGTGTGGGGAATATG GAATA
2018	pIMAY-Down-sRNA345-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGCCTTCAG TACATTATATAACCTTTGT
2031	pIMAY-Up-ncRNA2-F	CGACTCACTATAGGGCGAATTGGAGCTCGGCGTTCAATG GACTCTGTT
2032	pIMAY-Up-ncRNA2-R	GCGTATGGACCTAGGTATATCTTTTCATCTGTCCGATTT TTTGA

2033	pIMAY-Down-ncRNA2-F	ACCCACAACCTAGGTATATCTTGTGCTTCTCAATGATA CAATG
2034	pIMAY-Down-ncRNA2-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTCACCACCC AGTCATCAACA
2038	pIMAY-Up-ncRNA3-F	CGACTCACTATAGGGCGAATTGGAGCTCTCGGTTGCACA TACAGCTTT
2039	pIMAY-Up-ncRNA3-R	GCGTATGGACCTAGGTATATAAAAGTTTGAAGGTGATAAT GTACATG
2040	pIMAY-Down-ncRNA3-F	ACCCACAACCTAGGTATATAAACACTTTGCCCAACTTG C
2041	pIMAY-Down-ncRNA3-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTTTTACGGG TCTGTTTTCTAATTTGA
2066	Up-Sau5949-F	CGACTCACTATAGGGCGAATTGGAGCTCTCGTAATAATC GTGTGGCCA
2067	Up-Sau5949-R	GCGTATGGACCTAGGTATATACAATGTTATACTAAATAC CTTTGA
2068	Down-Sau5949-F	ACCCACAACCTAGGTATATGTACGAAAAAACACTTATG ATTGTATGT
2069	Down-Sau5949-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCACGGTAATT CAATCTATAGGTCTTGT
2119	pIMAY-Up-ssrS-F	CGACTCACTATAGGGCGAATTGGAGCTCACTCGTAAAGA TATGGATGCTT
2120	pIMAY-Up-ssrS-R	GCGTATGGACCTAGGTATATTATCTTATGATGTTATATT ACCACATAATT
2121	pIMAY-Down-ssrS-F	ACCCACAACCTAGGTATATTCTATCGATACGCAAGACT TTGTC
2122	pIMAY-Down-ssrS-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGGTGGCATT TGTCTTTTCG
2126	pIMAY-Up-sprF2-F	CGACTCACTATAGGGCGAATTGGAGCTCTGGATGGATTA AGAGGTTCGTGT
2127	pIMAY-Up-sprF2-R	GCGTATGGACCTAGGTATATCCACTATAATGAAGCATGC CTC
2128	pIMAY-Down-sprF2-F	ACCCACAACCTAGGTATATTGTCGTCTTTTTACATTTT TATAGTAAC
2129	pIMAY-Down-sprF2-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCACGCTCTAT TGACCCACCAA
2140	pIMAY-Up-sprA1As1-F	CGACTCACTATAGGGCGAATTGGAGCTCACTAACAAATA ATACACCAGCAGCT
2141	pIMAY-Up-sprA1As1-R	GCGTATGGACCTAGGTATATCACAGTCACTTGCTTCTGA TAAGTTA
2142	pIMAY-Down-sprA1-F	ACCCACAACCTAGGTATATGTGAGGGGATTGGTGTATA AGT
2143	pIMAY-Down-sprA1-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCGATTTATA TGAAGTACAATGTGAAAGG
2147	pIMAY-Up-sprF3-F	CGACTCACTATAGGGCGAATTGGAGCTCATGCAGATAGT ACACACCTGATTG
2148	pIMAY-Up-sprF3-R	GCGTATGGACCTAGGTATATCCAACTTTCCATACAGCAG AAAATAC
2149	pIMAY-Down-sprF3-F	ACCCACAACCTAGGTATATCGATAAACAGTTGAGTGAC ATACCC
2150	pIMAY-Down-sprF3-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCAGAGAACGG ATATACAATTGATAAAGAAGA
2161	pIMAY-Up-SprF1-F	CGACTCACTATAGGGCGAATTGGAGCTCGGCGCTTTACT TCCAACCTGT
2162	pIMAY-Up-SprF1-R	GCGTATGGACCTAGGTATATCACACCATAATATAAATAT CAAATAGACGG
2163	pIMAY-Down-SprF1-F	ACCCACAACCTAGGTATATTAAAAAGTCAGTACCGAAG CACT
2164	pIMAY-Down-SprF1-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCAACAATGT GCTGAGGAAGAGT

2168	pIMAY-Up-SprY3-F	CGACTCACTATAGGGCGAATTGGAGCTCAGATTAGAAGCGGCATTGC
2169	pIMAY-Up-SprY3-R	GCGTATGGACCTAGGTATATGTAAATAGAAAGCAGGTATGTAACGC
2170	pIMAY-Down-SprY3-F	ACCCACAACCTAGGTATATAACAGGCAGGTACTACGGTA
2171	pIMAY-Down-SprY3-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGAATCTCTTCGGCAACTTTG
2175	pIMAY-Up-SprX1-F	CGACTCACTATAGGGCGAATTGGAGCTCAACAGTTGGAA GTAAAGCGC
2176	pIMAY-Up-SprX1-R	GCGTATGGACCTAGGTATATCTTGAATACGTCTAGAAAAGATTATAACAT
2177	pIMAY-Down-SprX1-F	ACCCACAACCTAGGTATATTTATGACTTTAGCATTCCCGTATAATAGT
2178	pIMAY-Down-SprX1-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCACTCATTTTTAGGAATTTTCGCAA
2182	pIMAY-Up-SprY1-F	CGACTCACTATAGGGCGAATTGGAGCTCAACACACCATCGTTTGTTC
2183	pIMAY-Up-SprY1-R	GCGTATGGACCTAGGTATATACATATTCAATCAAGACAT TGCTT
2184	pIMAY-Down-SprY1-F	ACCCACAACCTAGGTATATATCAGTTAGGATGAAAAAGTGGAT
2185	pIMAY-Down-SprY1-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTACACACCA TCATTCAGCGA
2189	pIMAY-Up-SprX2-F	CGACTCACTATAGGGCGAATTGGAGCTCAACGGAACAAATGAACGTGA
2190	pIMAY-Up-SprX2-R	GCGTATGGACCTAGGTATATATCATAACAAAAAACTAGCCGAAG
2191	pIMAY-Down-SprX2-F	ACCCACAACCTAGGTATATTTAGCATTCCCGTATAACAGTTTAC
2192	pIMAY-Down-SprX2-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCATGCCCTATTTTATTTGTTGATGA
2196	pIMAY-Up-SprY2-F	CGACTCACTATAGGGCGAATTGGAGCTCAGCGTTATTAA GCAAGCAACT
2197	pIMAY-Up-SprY2-R	GCGTATGGACCTAGGTATATGTAAGATTCCTTATAATTAATGTAGCAAAA
2198	pIMAY-Down-SprY2-F	ACCCACAACCTAGGTATATTTATGTTATAGCTAGCCTTCGGG
2199	pIMAY-Down-SprY2-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCATGCAACGACTGATAAACCG
2201	pIMAY-Up-RsaH-F	CGACTCACTATAGGGCGAATTGGAGCTCTTAAACGGACC ACTAGCTGA
2202	pIMAY-Up-RsaH-R	GCGTATGGACCTAGGTATATGGTACACCTTTATTATAACTTATATCATTT
2203	pIMAY-Down-RsaH-F	ACCCACAACCTAGGTATATTTAGTGGACCCGTACGTTAA TC
2204	pIMAY-Down-RsaH-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTTGCTTTTGTAGGTGCTTGT
2208	pIMAY-Up-hfq-F	CGACTCACTATAGGGCGAATTGGAGCTCGGTGAAATCAT AAGCGGTGAC
2209	pIMAY-Up-hfq-R	GCGTATGGACCTAGGTATATCTGTGCGACTCCTTTTACTTAATC
2210	pIMAY-Down-hfq-F	ACCCACAACCTAGGTATATACGCTTCATATAAAGGTCCGAGT
2211	pIMAY-Down-hfq-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCAACATAATATTTGCGATCTACACG
2215	pIMAY-Up-sau5971-F	CGACTCACTATAGGGCGAATTGGAGCTCAATTACTTCTTCAAACCTAGCTTATTTCCG
2216	pIMAY-Up-sau5971-R	GCGTATGGACCTAGGTATATACTAGATAGTTTATACTTTTGGTCTGTTG

2217	pIMAY-Down-sau5971-F	ACCCACAACCTAGGTATATGAAGAGCTATGCATTTTAT TAAAAAT
2218	pIMAY-Down-sau5971-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCGAAGCAGA TTTTATTAAGTGTGT
2222	pIMAY-Up-00009-F	CGACTCACTATAGGGCGAATTGGAGCTCTGTGAACGCAG ATACAATGT
2223	pIMAY-Up-00009-R	GCGTATGGACCTAGGTATATACCCCATTAACCACAAAC T
2224	pIMAY-Down-00010-F	ACCCACAACCTAGGTATATGAACGCATTTTCATTATAGC AACAA
2225	pIMAY-Down-00010-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGCATCCAA CGATCATTTGAT
2228	pIMAY-Up-03030-F	CGACTCACTATAGGGCGAATTGGAGCTCCTTGTTCCTT AATTGTTGTACCT
2229	pIMAY-Up-03030-R	GCGTATGGACCTAGGTATATAGCACGCAATGATTTAAAG GAT
2230	pIMAY-Down-03031-F	ACCCACAACCTAGGTATATTCCTGAAAATTTGTATAA AGATTTAAGTC
2231	pIMAY-Down-03031-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGTCACGTCC TACAAACAAGT
2234	pIMAY-Up-01263-F	CGACTCACTATAGGGCGAATTGGAGCTCGCTACATTTGA AGTGAACGC
2235	pIMAY-Up-01263-R	GCGTATGGACCTAGGTATATGGATAGAAAACCAATCATC TTTATAGG
2236	pIMAY-Down-01264-F	ACCCACAACCTAGGTATATAATAAAAAAGAAGAGAAGA TGTAACACA
2237	pIMAY-Down-01264-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCGAGTTTGT CTTGTGCTTCC
2343	<i>isrR</i> amplification-F	CTGCAGGTCGACTCTAGAGGTGTGCGATTTTGAACCTGG A
2344	<i>isrR</i> amplification-R	CCATTCAGGCTGCGCAACTGGCGGTCATGCTATGGGATC A
2363	pIMAY-Up-S808-F	CGACTCACTATAGGGCGAATTGGAGCTCTGCCCCACCTA ATCAGATAT
2364	pIMAY-Up-S808-R	GCGTATGGACCTAGGTATATACCGAGTCATTTCAAGAAT G
2365	pIMAY-Down-S808-F	ACCCACAACCTAGGTATATGATAACCGCATCTTAACTG A
2366	pIMAY-Down-S808-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTCGTTTGCT AGAATAATTGCT
2379	pIMAY-Up-S596-F	CGACTCACTATAGGGCGAATTGGAGCTCTTGATGAAGAA CAATTAACAGCA
2380	pIMAY-Up-S596-R	GCGTATGGACCTAGGTATATCGTTTTATAAAAGCAGTAA ACCCT
2381	pIMAY-Down-S596-F	ACCCACAACCTAGGTATATGATGTTCTATGTGGTATTG ATAATCA
2382	pIMAY-Down-S596-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGCGACAAA TTTCTAAGCCA
2387	pIMAY-Up-S204-F	CGACTCACTATAGGGCGAATTGGAGCTCGCTTTAACTGC CATCGTTAC
2388	pIMAY-Up-S204-R	GCGTATGGACCTAGGTATATATAACCACATCACATAAAT TGAGTTC
2389	pIMAY-Down-S204-F	ACCCACAACCTAGGTATATGTCTTAGTAAATCATAACGT TCTATGT
2390	pIMAY-Down-S204-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCTATTGAATG CCGACAGACTC
2395	pIMAY-Up-RsaC-F	CGACTCACTATAGGGCGAATTGGAGCTCCCTGTTGGTCA AGATCCTCA
2396	pIMAY-Up-RsaC-R	GCGTATGGACCTAGGTATATTTGTTGATGTGTGGCCTAAA A

2397	pIMAY-Down-RsaC-F	ACCCACAACCTAGGTATATCCTAAAAATAAAAGGGATTG ATGAAAAGC
2398	pIMAY-Down-RsaC-R	CCTCACTAAAGGGAACAAAAGCTGGGTACCCGAAACCTG CACCTAAAAACA
2499	+1 <i>isrR</i> for pRMC2 F	GATAGAGTATAATTAATAAAGCGTTGAAAATGATTATC AATACCAC
2500	+1 <i>isrR</i> for pRMC2 R	TCAGATCTGTTAACGGTACCAAAATAGTAAAAAACAAAA GCAGTAAAC
2507	pIMAY-locus2 R	CGCACATTGAAATGATGTGTGAGCAGCAATGATTTAAA GGAT
2508	pIMAY-locus2 F	TTGATCCCATAGCATGACCGTCACTGAAAATTTGTATAA AGATTTAAGTC
2509	pIMAY-locus3 R	CGCACATTGAAATGATGTGTGGGATAGAAAACCAATCAT CTTTATAGG
2510	pIMAY-locus3 F	TTGATCCCATAGCATGACCGAATAAAAAAGAAGAGAAGA TGTAACACA
2511	<i>isrR</i> for pIM-locus2 or 3 F	CACACATCATTTCAATGTGCG
2512	<i>isrR</i> for pIM-locus2 or 3 R	CGGTCATGCTATGGGATCAA
2534	Linearization pCN34 R	CTCGGTACCCGGGGATCCTC
2535	Linearization pCN34 F	CCGTCGTTTTTACAACGTCGTG
2538	$\Delta tetR$ from pRMC2- <i>isrR</i> R	CCACAGACAAAATCACAGATACTAGTTTTTTTTATTTGGATC CCC
2539	$\Delta tetR$ from pRMC2- <i>isrR</i> F	TATCTGTGATTTGTCTGTGGAAGCAGCATAACCTTTTTTC CG
2546	pCN38- <i>isrR</i> ( <i>isrR</i> promoter-terminator) F	ATGGTCATAGCTGTTTCTGATAAAAGCAGTAAACCCTT ACGA
2547	pCN38- <i>isrR</i> ( <i>isrR</i> promoter-terminator) R	ATAATCATCCTCCTAAGGTACCCGGATGTTCTATGTGGT ATTGATAATC
2552	PCR of GFP with an RBS (from pCM11) F	GGGTACCTTAGGAGGATGATTA
2553	PCR of GFP with an RBS (from pCM11) R	CAGGAAACAGCTATGACCATG
2554	PCR of <i>rrnB</i> terminator from pECTO F	CTGTTTCCTGAGTAGGGAAGTCCAGGC
2555	PCR of <i>rrnB</i> terminator from pECTO R	ACGACGTTGTAAAACGACGGAATTAATGACAATCCTACT CAGGAGAG
2573	Fur motif 1 mutagenesis F	TAGTTTCTAATTGACAAATGAAAGACACTAATGTATAATA GTAGTTGAAAA
2574	Fur motif 1 mutagenesis R	TTTTCAACTACTATTATACATTAGTGTCTTTCATTGTCA ATTAGAAACTA
2575	Fur motif 2 mutagenesis F	CTAATGTATAAATAGTAGTTGTTTTTGATTATCAATACCA CATAGAACATC
2576	Fur motif 2 mutagenesis R	GATGTTCTATGTGGTATTGATAATCAAAAACAACACTACTA TTATACATTAG
2601	pCN34-sGFP F	AGCAAAGGAGAAGAAGACTTTTCAC
2600	pCN34-sGFP R	TTAGTTAATTATAACTAATTAATAAATGAGAAGTAAAC
2605	<i>isrR</i> $\Delta$ CRR1 F	CCACATAGAACATACAACGTTTCGTTCTTGTGGAT
2606	<i>isrR</i> $\Delta$ CRR1 R	ACGAAACGTTGTATGTTCTATGTGGTATTGATAATC
2617	<i>narG</i> 5'UTR-F for pCN34-GFP F	TTACTTCTCATTTTTTAATTAGTTATAATTAATAAAAAAG CAATAGTCTTGGGCATTTT
2618	<i>narG</i> 5'UTR-R for pCN34-GFP R	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTGCTTCTA CTTTTACTTTCTAGGATCG

2619	nasD 5'UTR-F for pCN34-GFP F	TTACTTCTCATTTTTAATTAGTTATAATTAACATAACCT TTTTTTGAAATAAATATTATG
2620	nasD 5'UTR-R for pCN34-GFP R	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTGCTGCGC TCTAATATTTCTTCGATT
2621	gltB2 5'UTR-F for pCN34-GFP F	TTACTTCTCATTTTTAATTAGTTATAATTAACATAATACA TTAAAAATTTAAAAATGAAAAA
2622	gltB2 5'UTR-R for pCN34-GFP R	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTGCTTATA AATTGCATGACTGTAAGAA
2631	<i>isr</i> ΔCRR2 F	CATTTTTCAAATATTCTTTTTATATGCCCGTAAAAGACAA
2632	<i>isr</i> ΔCRR2 R	GGCATATAAAAAGAATATTTGAAAATGACCAATCCAAC
2667	<i>isr</i> ΔCRR3 F	CCCTTTTATATGGTAAAAGACAATATACGTTATAACAAC G
2668	<i>isr</i> ΔCRR3 R	ATTGTCTTTTACCATATAAAAAGGGGAATATTTGAAAATG A
2694	fdhA 5'UTR-F for pCN34-GFP F	TTAATTAGTTATAATTAACATAAAATTCATCTGAAAGAT GTGTG
2695	fdhA 5'UTR-R for pCN34-GFP R	AAAAGTTCTTCTCCTTTGCTATCAAGTGAACCACCAAA TG
<b>Northern blot probes</b>		
2452	IsrR	TCTTTTACGGGCATATAAAAAGGGG
2614	<i>S. lugdunensis</i> IsrR	TCTTTTAAAGGGCATATAAAAAGGGG
2526	<i>fdhA</i> mRNA	TGGATACGCACCAGTACCAG
2627	<i>ssrA</i> (tmRNA)	CTTCAAACGGCAGTGTTTAGC
2696	<i>gltB2</i> mRNA F	ACGGTTATTGTTATCGGGCT
2697	<i>gltB2</i> mRNA R	AAGCGCCATAACTCATAACCA
2922	IsrR EMSA	CAATCCAACAAGAACGAAACGTTG
<b>5'/3'RACE mapping</b>		
2728	IsrR RT	CGTATATTGCTTTTTACGGGC
2729	IsrR 5'3' junction PCR F	CAACGTTTTATAAAAAGCAGTAAACCC
2730	IsrR 5'3' junction PCR R	CAATCCAACAAGAACGAAACGTT
2731	IsrR 5'3' junction nested PCR F	CGACACTTTAGGTTTACTGCTTTTG
2732	IsrR 5'3' junction nested PCR R	GGGATGTTCTATGTGGTATTGATAATC
<b>SHAPE. PCR for DNA template</b>		
-	IsrR F	TAATACGACTCACTATAGTTGAAAATGATTATCAATACC ACATAG
-	IsrR R	ACAAAAGCAGTAAACCTAAAGTG
-	FdhA F	TAATACGACTCACTATAGAATTCTATCTGAAAGATGTGT GG
-	FdhA R	GGTACAAAAGTATCTTGTGATT
-	GltB2 F	TAATACGACTCACTATAGACTTTAGCACATATTACTTTG TATTG
-	GltB2 R	CGATAACAATAACCGTAAGCATG
-	NarG F	TAATACGACTCACTATAGAAAAGCAATAGTCTTGGGCATT TTAA
-	NarG R	TTGTTCTTACTTCTTTATCGTGGCT
-	NasD F	TAATACGACTCACTATAGATTAGAAAAGCTTAATGATTCC AATG
-	NasD R	ATAGTTTGGATAAGGTTCTTTACCT



<b>SHAPE. Reverse transcription</b>		
-	IsrR	CCTAAAGTGTTCGTAAGGG
-	FdhA	TAATAAAATTC AAGTAAATTCGTACC
-	GltB2	AATCCTACAACGATAATGTTAAC
-	NarG	TTGTTCTTACTTCTTTATCGTGGCT
-	NasD	ATAGTTTGGATAAGGTTCTTTACCT
-	IsrR competitor	ACAAAAGCAGTAAA

\* F, forward primer; R, reverse primer.

Table S4. Fitness library composition

Allele	Library 1	Library 2	Library 3
<i>ΔrnallI::tag004</i>	SAPhB618	SAPhB618	SAPhB618
<i>ΔrsaOG::tag009</i>	SAPhB347	SAPhB347	SAPhB347
<i>ΔrsaG::tag011</i>	SAPhB349	SAPhB349	SAPhB349
<i>Δteg147::tag018</i>	SAPhB368	SAPhB368	SAPhB368
<i>ΔrsaB::tag025</i>	SAPhB380	SAPhB380	SAPhB380
<i>ΔrsaD::tag026</i>	SAPhB682	SAPhB682	SAPhB682
<i>Δteg116::tag030</i>	SAPhB386	SAPhB386	SAPhB386
<i>Δsau85::tag038</i>	SAPhB397	SAPhB397	SAPhB397
<i>Δsau6353::tag042</i>	SAPhB402	SAPhB402	SAPhB402
<i>ΔrsaE::tag045</i>	SAPhB404	SAPhB404	SAPhB404
<i>Δssr42::tag050</i>	SAPhB412	SAPhB412	SAPhB412
<i>Δteg155::tag053</i>	SAPhB415	SAPhB415	SAPhB415
<i>sprF3::tag070</i>	SAPhB960	SAPhB961	SAPhB962
<i>sRNA334::tag073</i>	SAPhB862	SAPhB863	SAPhB864
<i>rsaA::tag075</i>	SAPhB943	SAPhB944	SAPhB945
<i>sau76::tag076</i>	SAPhB890	SAPhB891	SAPhB962
<i>rsaO1::tag077</i>	SAPhB883	SAPhB884	SAPhB885
<i>teg16::tag080</i>	SAPhB865	SAPhB866	SAPhB867
<i>sRNA287::tag085</i>	SAPhB871	SAPhB872	SAPhB873
<i>sRNA71::tag086</i>	SAPhB874	SAPhB875	SAPhB876
<i>sRNA209::tag093</i>	SAPhB907	SAPhB908	SAPhB909
<i>teg106::tag095</i>	SAPhB899	SAPhB900	SAPhB901
<i>sRNA260::tag096</i>	SAPhB921	SAPhB922	SAPhB947
<i>sRNA345::tag097</i>	SAPhB910	SAPhB911	SAPhB912
<i>ncRNA2::tag099</i>	SAPhB932	SAPhB933	SAPhB934
<i>ncRNA3::tag100</i>	SAPhB940	SAPhB941	SAPhB942
<i>ssrS::tag107</i>	SAPhB954	SAPhB955	SAPhB956
<i>sprF1::tag110</i>	SAPhB1006	SAPhB1007	SAPhB1008
<i>sprX2::tag111</i>	SAPhB974	SAPhB975	SAPhB976
<i>sprY2::tag112</i>	SAPhB978	SAPhB979	SAPhB980
<i>sprY3::tag113</i>	SAPhB957	SAPhB958	SAPhB959
<i>sau41::Tag115</i>	SAPhB901	SAPhB902	SAPhB903
<i>sau5949::tag117</i>	SAPhB948	SAPhB949	SAPhB950
<i>sprF2::tag118</i>	SAPhB966	SAPhB967	SAPhB998
<i>sprB::tag121</i>	SAPhB1031	SAPhB1032	SAPhB1033
<i>rsaC::tag133</i>	SAPhB1242	SAPhB1243	SAPhB1244
<i>S204::tag134</i>	SAPhB1234	SAPhB1235	SAPhB1236
<i>S596::tag135</i>	SAPhB1231	SAPhB1232	SAPhB1233
<i>S808::tag137</i>	SAPhB1239	SAPhB1240	SAPhB1241
<i>locus3::tag139</i>	SAPhB1015	SAPhB1016	SAPhB1017
<i>locus2::tag140</i>	SAPhB1012	SAPhB1013	SAPhB1014
<i>locus1::tag141</i>	SAPhB1009	SAPhB1010	SAPhB1011
<i>sau5971::tag142</i>	SAPhB1018	SAPhB1019	SAPhB1020
<i>sprA1::tag144</i>	SAPhB976	SAPhB977	SAPhB996
<i>sprX2::tag145/sprX1::tag149</i>	SAPhB1027	SAPhB1028	SAPhB1029
<i>sprX1::tag146</i>	SAPhB1003	SAPhB1004	SAPhB1005
<i>rsaH::tag147</i>	SAPhB971	SAPhB972	SAPhB973
<i>sprY1::tag148</i>	SAPhB1021	SAPhB1022	SAPhB1023

**Table S5. IsrR sequences in *Staphylococcus* genus**

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>NC\_007795 (*Staphylococcus aureus* NCTC 8325)  
 AAAATAGCGTAATCATGCGTTTTATTACTATTCTTAAAAAATATTCAAAAAAGTTTTAGTTTCT**TAAT**  
**TGACAATGATTCTCACTAATGTATAATAGTAGTTGAAAATGATTATCAATACCACATAGAACATCCCCC**  
**CCACAACGTTTCGTTCTTGTGGATTGGTCATTTTCAAATATTCCCCTTTTATATGCCCGTAAAAGACA**  
**ATATACGTTATAACAACGTTTTATAAAAAGCAGTAAACCCCTTACGACACTTTAGGTTTACTGCTTT**

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>LS483491.1 (*Staphylococcus auricularis* NCTC12101)  
 ACTCTTCATTATAAAATAATCTCAAATAAATGTATAGCAAAAAAAGTGAAAAGAAAGTTGCAGGATT**TAA**  
**TTGACAATAATTCTCAGTCATGTATAATGTAGTTGGAAAATGATTATCAATACCAAGTAAGATCTTCCCC**  
**CCCACACTTATATGTTCTTATTGGATTGATCATTTTCAATATCCCTTTTATATTCCCGTAAAAGA**  
**CTAACGTTGTAATCAACGTGTGAGATAAAGACCGTTTTTACGAATGTAAAACGGTCTTTATAATAAAC**  
**A**

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>NZ\_CP016760 (*Staphylococcus carnosus* LTH 3730)  
 TTAAAGATACTCATTTTTGTGTAAATAACTTACAGAAAAAGAAAAATTCAAAAAAGTTACTTTTCT**TAATT**  
**GACAATCATTCTCAATAATGTATAATAGTACTTGAAAATGATTATCAATACCAAATGAGACATCCCCC**  
**ACAATTTTCGTTCTTATAGTATTGGTCATTTTCAATATCCCTTTTATATATGCCCGTAAATACTGACGTT**  
**ATGGTTATACATAACGTGACATACTTACCGATTTTGTCTAGTAAATGCAAAATCGGTTTT**

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>NC\_002976 (*Staphylococcus epidermidis* RP62A)  
 TTTAGAAAAAGTCACTCTAGCATTATCATTGTATTTAAGTTAAATGGAATAAAATATATATTTCT**TAATT**  
**GACAATCATTATCAATCATGTATAATGATAATTGAAAATGATTATCAATACCAATGAAAAACATTTCCC**  
**CCCACATAACAAGTTGTTCTTTTGGATTGGTCATTTTCAACTATCCCTTTTATATGCCCGTAAAAGACT**  
**AACGTTAAGAAATGACGTTTCAATAAAAAGCAGTAGACCTTTGACACTTGAGGTCTGCTGTTTT**

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>NC\_007168 (*Staphylococcus haemolyticus* strain Sh29/312/L2)  
 AAATAGAGAATGGATTAAAAATGTAATGATATAATCAAATTTAAATGAGAATTTTTCGCAATTT**TAATT**  
**GACAATGATTATCAATGATGTATAATAGTATTGAAAATGATTATCAATACCGAATAAGACAATTTCCCC**  
**CCACATATATTTTCGTTCTTAAAGGATTGGTCATTTTCAAGTTATTCCCCTTTTATATGCCCGTAAAAGA**  
**CTAACGTTAAGTTTCAAACCTACTTTAAACGTTTTTAATAAAAAGCAGTGAATCTAATGCCGAGGT**

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>NZ\_CP008747 (*Staphylococcus hyicus* strain ATCC 11249)  
 ACCTCACATCTATCATATTTCAAACGAGCCAGATAAAAGTTTTTTAAGAAAAATACGAGATTT**TAAT**  
**TGACAATCATTCTCAATAAGGTATAATGTAAATTGAAAATGATTCTCAATCGTAACGCCCCCCACTACA**  
**ATTCGTTCTTTTTGATTGAGGCATTTTCAAGTACTATCCCTTTTATATGCCCGTATAAAAAAATAGTC**  
**GTTTTGATTAAACGTTAAATTTAAGCTGCTACACTTATGTGTAGTAGCTCCTTTTTTGT**

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>NZ\_CP020768 (*Staphylococcus lugdunensis* strain C\_33)  
 CACCAACCATAGTCTAAGATTTATGATTAACTTTCAAAAAATTTATCATAAAAAATTTAGTTTTCT**TAATT**  
**GACAATCATTATCAATGATGTATAATAATAATTGAAAATGATTATCAATACCAAATAGAACTCCCCCA**  
**CATATTCGTTCTTATGGATTGATCATTTTCAAGTTTCCCTTTTATATGCCCTTAAAAGACTAACGTAA**  
**GCTTACAATAACGCTAAACGTGTACATAAAAAGCAGCTCCCTAATGGTAGCTGCTTT**

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>NC\_007350 (*Staphylococcus saprophyticus* ATCC 15305)  
 TGATTTTAAATCCCTTTTATTGTAATATATAGGTATTTCAAAAAATTTGAAAAAATTTATTCGAATTT**TAAT**  
**TGACAATCATTCTCGGTGTTGTATAATGTAAATTGAAAATGATTATCAATACCAAATAAGACATTTCCCC**  
**CCACACATATTTTCGTTCTTATTGGATTGATCATTTTCAAAAAATTCCTTTTATATGCCCGTAAAAG**  
**ACTAACGTTGCGAGACAACGTGAATATAAAAACCGTTTTTACATTGTAAAATCGGTTTTTTATAATAA**

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>NZ\_CP022046 (*Staphylococcus sciuri* SNUDS-18)  
 AACATGTTTCATTATATATGAGCATTACTATTTTATCATTTAAAAATAGTAAAAATTTTAAATAAC**TAATT**  
**GACATTCATTCTCAATTGGTTATAATAGTAAATTGAAAATGATTATCAATCAAATAAAGAGTTCCCTC**  
**TAAGTATAGATTGAACATTTTCTATAACCCCTTTTATGCCCATAAAAGTAAAATAGCCGCAGTGATTC**  
**TGTCCAAATCACTGTGGCTGTTTTTTTTTGTTTGGATTATTGAGGA**

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>NZ\_CP023497 (*Staphylococcus simulans* strain FDAARGOS\_383)  
 ATCAAATGATTAATTGTGACAAAAATATGGAGTTGAATAAAAAAGTGAAATTTATTTTGGATTTCT**TAATT**  
**GACAACCATTCTCACTAATGTATAATAGTAAATTGAAAATGATTATCAATCACAAATGAGACATCCCCC**  
**ACATTTTCGTTCTTAAATTGGATTGGTCATTTTCAAGTTTATCCCTTTTATATATGCCCGTAAATACTGAC**  
**GTTACTACTAAAAGTAACGTGACATACTTAAACCGATTCTCAATTAAGCAGAATCGGTTTTTTTTCT**

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>NC_020164 (Staphylococcus warneri SGI)  
TTCGTAATGTCAAAAATTGATGATGGTTAATAATTTATAAATAGTTCATCATTTCGTTATTTCTTAAT  
TGACAATGATTCTCATTCATGTATAATAATAGTTGAAAATGATTATCAATACCAAAAAGAACTTTCCCC  
CCCACATATATTTTCGTTCTTAAGGATTGGTCATTTTCATATAATCCCCTTTTATATGCCCGTAAAAGAC  
TAACGTTGAAAACGTTTTAATAAAGCAGTAGACCTTTAGACACTTGAGGTTTACTGCTTT  
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NCTC8325 *isrR* orthologs were searched within the Firmicutes phylum using GLASSgo (version 1.5.0 RNA tool) set with default parameters (11). As no sequence of *S. auricularis* was present in the GLASSgo dataset, the presence of an *isrR* sequence within this specie was search and found using the NCBI DNA sequence repository. An *isrR* ortholog (red characters) is present in all members of the staphylococcus genus. A sequence of one selected representative of each *Staphylococcus* genus group is shown. The 100 nt upstream sequences are shown (black characters). All sequences contain putative Fur boxes (characters in bold).

**Table S6. Proteins containing an Fe-S cluster in *S. aureus***

Symbol	Fe-S cluster-containing protein
NirB	Nitrite reductase [NAD(P)H] large subunit
BioB	Biotin synthase
FdhL	Putative formate dehydrogenase
PflA	Pyruvate formate-lyase-activating enzyme
SdhB	Succinate dehydrogenase iron-sulfur subunit
MiaB-like	Uncharacterized protein
MiaB	tRNA-2-methylthio-N(6)-dimethylallyl-adenosine synthase
Nth	Endonuclease III
QueE	7-carboxy-7-deazaguanine synthase
RlmN	Probable dual-specificity RNA methyltransferase
NirD	Nitrite reductase [NAD(P)H] small subunit
MoaA	GTP 3',8-cyclase
HemW	Heme chaperone
LipA	Lipoyl synthase
NarH	Respiratory nitrate reductase beta subunit
GltB	Glu_synthase domain-containing protein
QueG	Epoxyqueuosine reductase
GltB	Glutamate synthase large subunit
Ferredoxin	Ferredoxin
SufA	Fe-S_biosyn domain-containing protein
AddB	ATP-dependent helicase/deoxyribonuclease subunit B
SdaA	L-serine dehydratase
GltD	Glutamate synthase subunit beta
MutY	Adenine DNA glycosylase
AcnA	Aconitate hydratase
RumA	RNA methyltransferase
NrdG	Anaerobic ribonucleoside-triphosphate reductase-activating protein
YfkB	Uncharacterized protein
Grx	Glutaredoxin domain-containing protein
NarG	Nitrate reductase
Nfu	NifU domain-containing protein
LeuC	3-isopropylmalate dehydratase large subunit
YhcC-like	Elp3 domain-containing protein

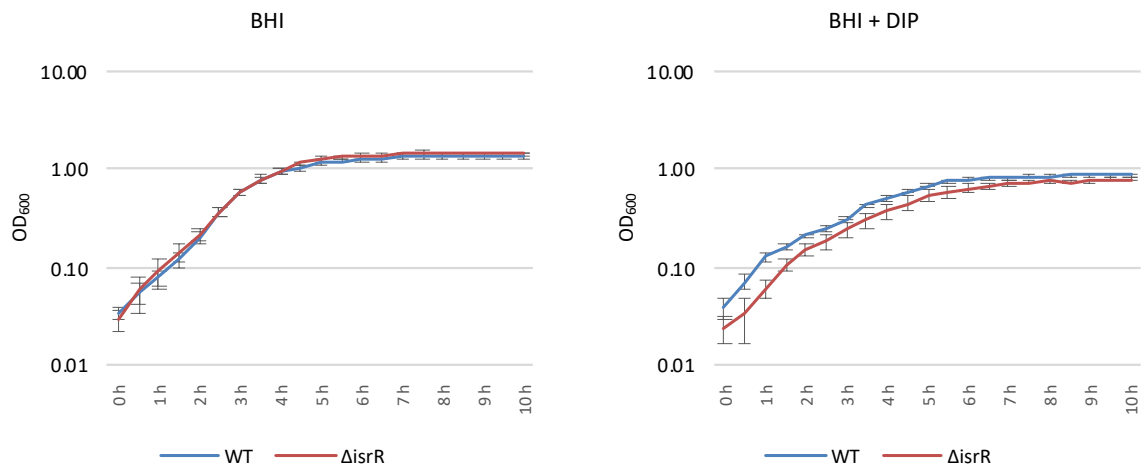
List was manually curated for *S. aureus* strain USA300 combining data from MetalPredator (12) and UniProt (13) web servers, and whether the protein possesses the conserved cysteine ligands. (Béatrice Py, personal communication).

**Table S7. IsrR functional analogs and their targets**

IsrR functional analog	Organism	RNA chaperone	mRNA targets and products	References
<b>FsrA</b>	<i>Bacillus subtilis</i>	FbpA, FbpB and FbpC	<b>sdhCAB*</b> , succinate dehydrogenase <b>citB*</b> , aconitase <b>gltAB*</b> , glutamate synthase <b>leuCD</b> , isopropyl malate dehydratase <b>ilvC</b> , ketol-acid reductoisomerase <b>cydA</b> , cytochrome bd ubiquinol oxidase <b>resA</b> , thiol-disulfide oxidoreductase <b>ctaO</b> , heme O synthase <b>cysH</b> , adenosine 5'-phosphosulfate reductase <b>lutABC*</b> , lactate oxidase <b>dctP*</b> , dicarboxylate permease	(14,15)
<b>Msrl</b>	<i>Mycobacterium tuberculosis</i>	-	<b>bfrA*</b> , bacterioferritin <b>hypF</b> , hydrogenase maturation factor <b>fprA</b> , NADPH-ferredoxin reductase <b>acnA</b> , aconitase	(16)
<b>NrrF</b>	<i>Neisseria meningitidis</i>	Hfq	<b>sdhCDAB*</b> , succinate dehydrogenase <b>petABC*</b> , cytochrome <i>bc<sub>1</sub></i> <b>gpxA</b> , glutathione peroxidase <b>tadA</b> , tRNA-specific adenosine deaminase <b>suhB</b> , extragenic suppressor protein <b>mgo</b> , malate:quinone oxidoreductase <b>hemO</b> , heme oxygenase	(17-19)
<b>PrrF1, PrrF2</b>	<i>Pseudomonas aeruginosa</i>	Hfq	<b>sodB*</b> , superoxide dismutase <b>sdhCDAB*</b> , succinate dehydrogenase <b>acnA*</b> , aconitase A <b>acnB*</b> , aconitase B <b>katA</b> , catalase <b>antABC*</b> , anthranilate dioxygenase <b>catBCA</b> , catechol dissimilatory complex	(20,21)
<b>RyhB</b>	<i>Escherichia coli</i>	Hfq	Selected targets: <b>acnA*</b> , aconitase A <b>acnB</b> , aconitase B <b>bfr*</b> , bacterioferritin <b>fdhF</b> , formate dehydrogenase H <b>fdoGHI</b> , formate dehydrogenase O <b>gltB*</b> , glutamate synthase large chain <b>mgo</b> , malate:quinone oxidoreductase <b>cydAB</b> , cytochrome d terminal oxidase <b>hyaA</b> , hydrogenase-1 small chain <b>katG</b> , catalase-peroxidase <b>narG</b> , nitrate reductase alpha-chain <b>narK</b> , nitrate/nitrite transporter <b>nirB</b> , nitrite reductase large subunit <b>sdhCDAB*</b> , succinate dehydrogenase <b>sodB*</b> , superoxide dismutase <b>mrsB*</b> , methionine sulfoxide reductase <b>oppB</b> , oligopeptide transport system permease protein	(22-29)  <a href="https://doi.org/10.1371/journal.pone.0063647">https://doi.org/10.1371/journal.pone.0063647</a>

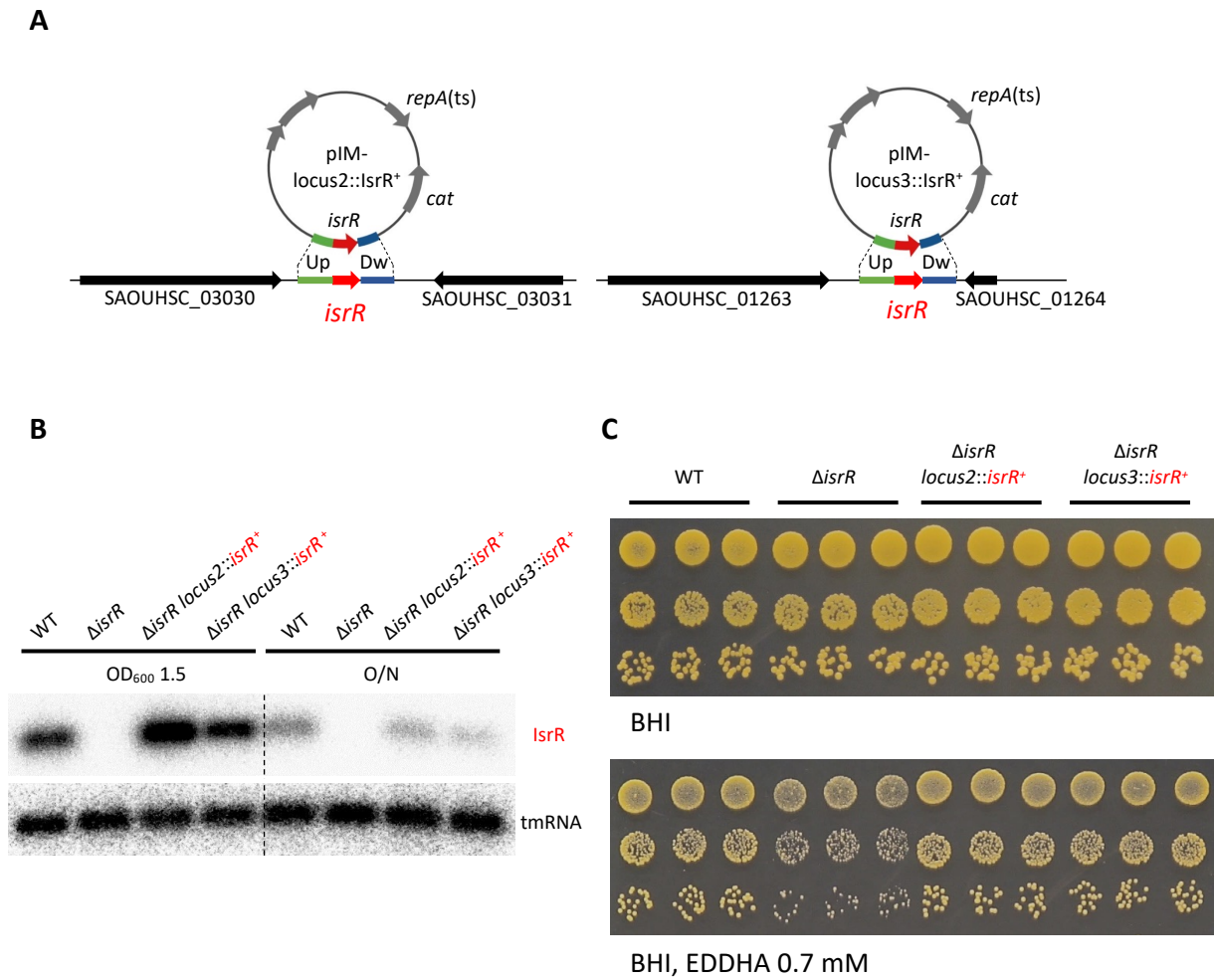
\*Validated targets. In red, shared functional targets with IsrR.

**Figure S1. Absence of *IsrR* is detrimental when iron is scarce**



Growth of *ΔisrR* and its parental HG003 strain. Cultures were grown in rich medium (BHI) or BHI with 2,2'-dipyridyl (DIP) 1.5 mM at 37°C under vigorous agitation in microtiter plates in a volume of 200μL. Incubation and OD<sub>600</sub> were obtained using CLARIOstar microplate reader. Error bars indicate the standard deviation from three independent biological samples (n=3).

**Figure S2. *isrR* complementation restores optimal growth in low-iron conditions**



A) Schematic representation of constructions for chromosomal ectopic complementation of  $\Delta isrR::tag135$  with insertion at locus2 (SAPhB1500) and locus3 (SAPhB1502). B) *IsrR* expression. Northern blot experiments with probes detecting *IsrR* and *tmRNA* (loading control). The indicated strains were sampled at  $OD_{600}$  1 and overnight cultures with DIP 1.25 mM ( $n=2$ ). C) Plating efficiency (10-fold serial dilutions) of indicated strains on BHI medium without (upper panel) or with (lower panel) EDDHA 0.7 mM. Three independent biological clones are shown for each strain ( $n=3$ ). For strains, see Table S1.



**Figure S3. IsrR 5'/3'RACE mapping and LocARNA secondary structure prediction**

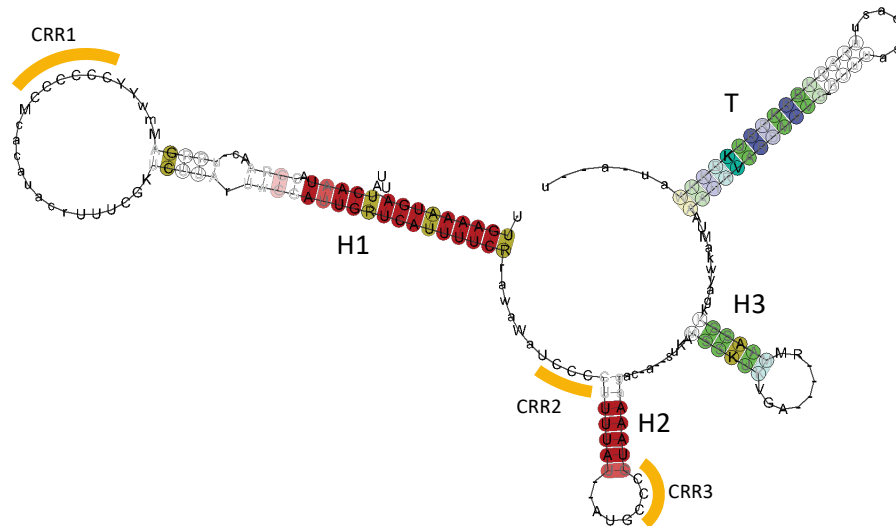
**A**

G<sup>6</sup>T<sup>6</sup> TGA<sup>6</sup> AA<sup>2</sup> TGA<sup>2</sup> TTATCAATACCACATAGAACATCCCCCCCACAACGTTTCGTTCTTGTTGGATTGGT  
 CATTTCAAATATCCCCTTTTATATGCCCGTAAAAGACAATATACGTTATAACAACGTTTATAAAAAGCAGT  
 AAACCCTTACGACACTTTAGGTTTACTGCTT<sup>1</sup> T<sup>1</sup> G<sup>1</sup> T<sup>6</sup> T<sup>2</sup> T<sup>1</sup> TTACTATTT

**IsrR** (174 nt):

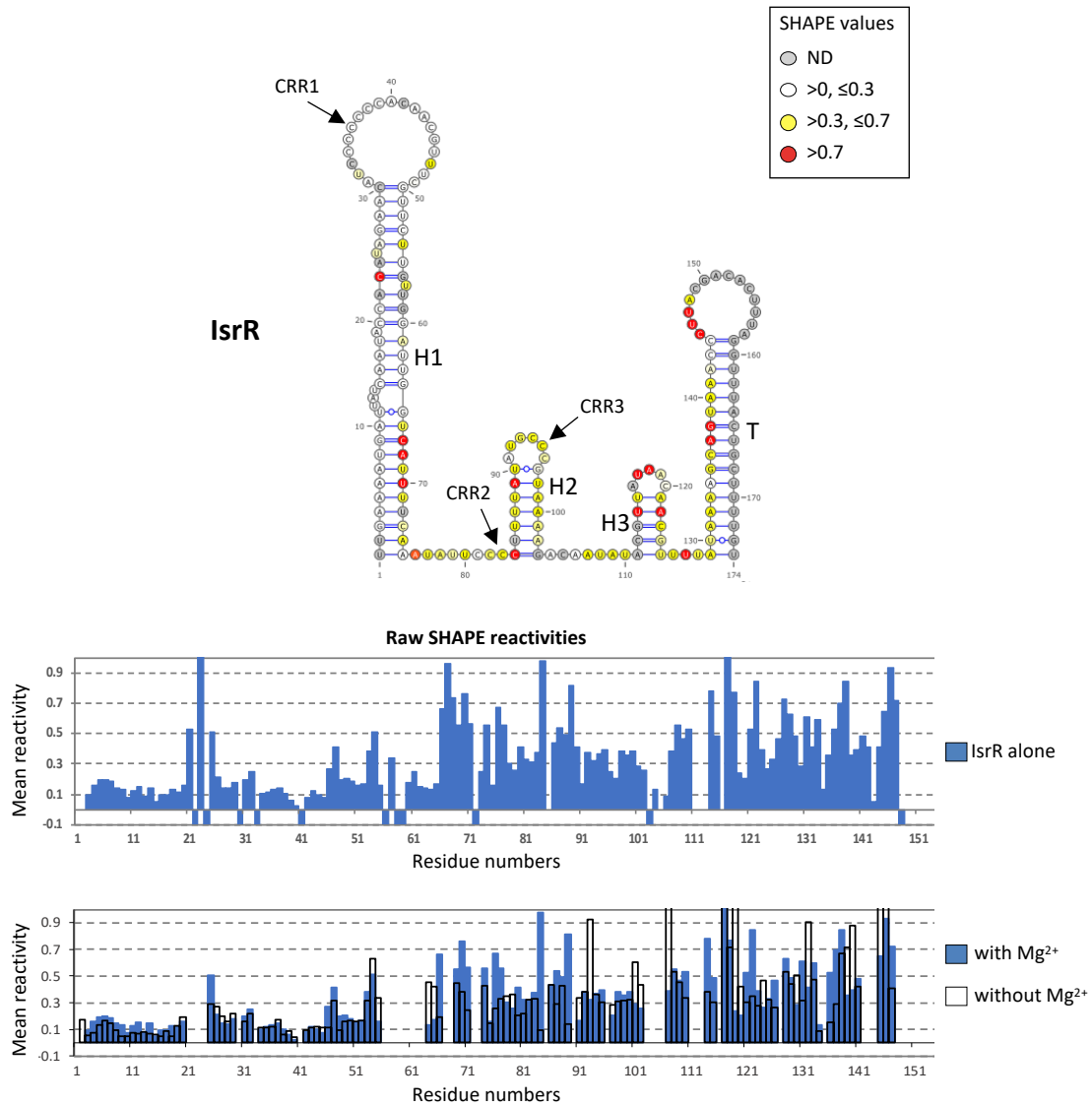
TTGAAAATGATTATCAATACCACATAGAACATCCCCCCCACAACGTTTCGTTCTTGTTGGATTGGTCATTTTC  
 AAATATCCCCTTTTATATGCCCGTAAAAGACAATATACGTTATAACAACGTTTATAAAAAGCAGTAAACCCT  
 TACGACACTTTAGGTTTACTGCTTTTGT

**B**



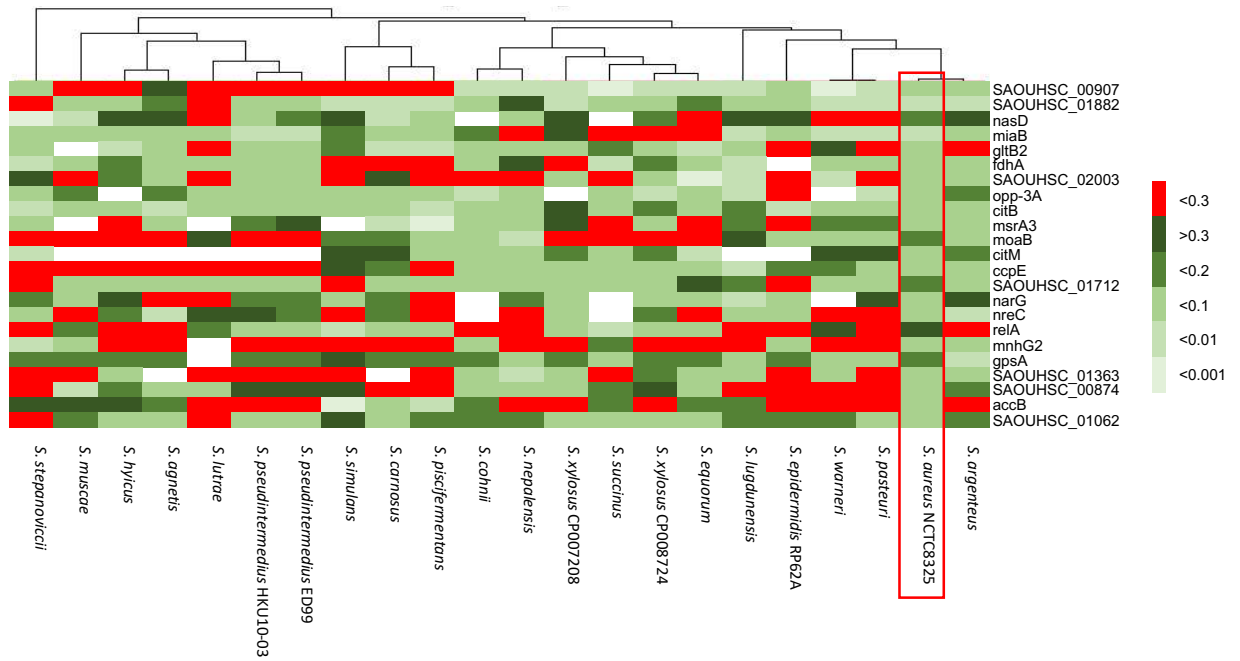
A) Mapping of IsrR extremities. Upper part, results from 5'/3'RACE mapping. Superscript numbers indicate the number of sequences ending with the corresponding nucleotide that were obtained. Lower part, IsrR sequence retained for the study. B) IsrR secondary structure predicted by LocARNA with 18 ortholog sequences for input (see Fig. 3A). LocARNA takes into account nucleotide covariations between different sequences restoring pairings to support the existence of stem-loop structures. Three stem loop structures (H1 to H3) and a rho-independent transcription terminator (T) are predicted. Three predicted C-rich regions (CRR1-3) are indicated. Colors indicate the number of base pairing types (red, 1; ochre, 2; green, 3; blue, 4; dark blue, 5; as per LocARNA parameters (30)) and hue shows sequence conservation (number of incompatible pairs: saturated, 0; medium, 1; light, 2).

**Figure S4. IsrR secondary structure obtained with IPANEMAP and reactivity profile**



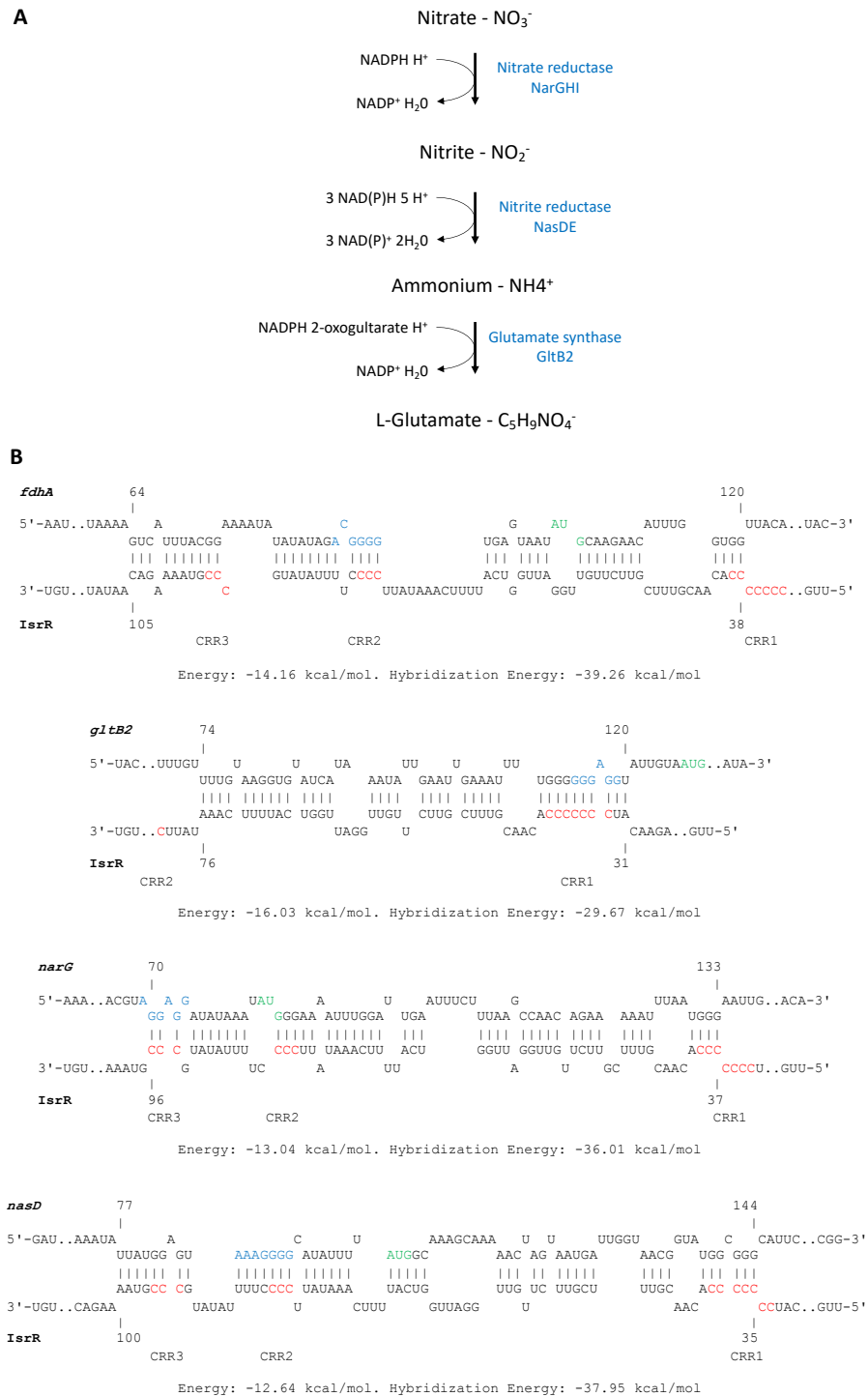
IsrR was probed with 1M7 at 37°C in 40 mM HEPES pH 7.5, 150 mM KCl with/without 5 mM  $MgCl_2$ . Top panel: IsrR structure predicted by IPANEMAP. White: low reactivity, yellow: moderate reactivity, red: high reactivity. Nucleotides in grey denote undetermined reactivity. Specific regions of the IsrR structure are indicated: Three stem loop structures (H1 to H3), a rho-independent transcription terminator (T) and three C-rich regions (CRR1 to CRR3). Middle panel: Reactivity profile of IsrR alone in the presence of  $Mg^{2+}$ . Bottom panel: Comparison of reactivity in presence or absence of  $Mg^{2+}$ .

Figure S5. IsrR putative targets



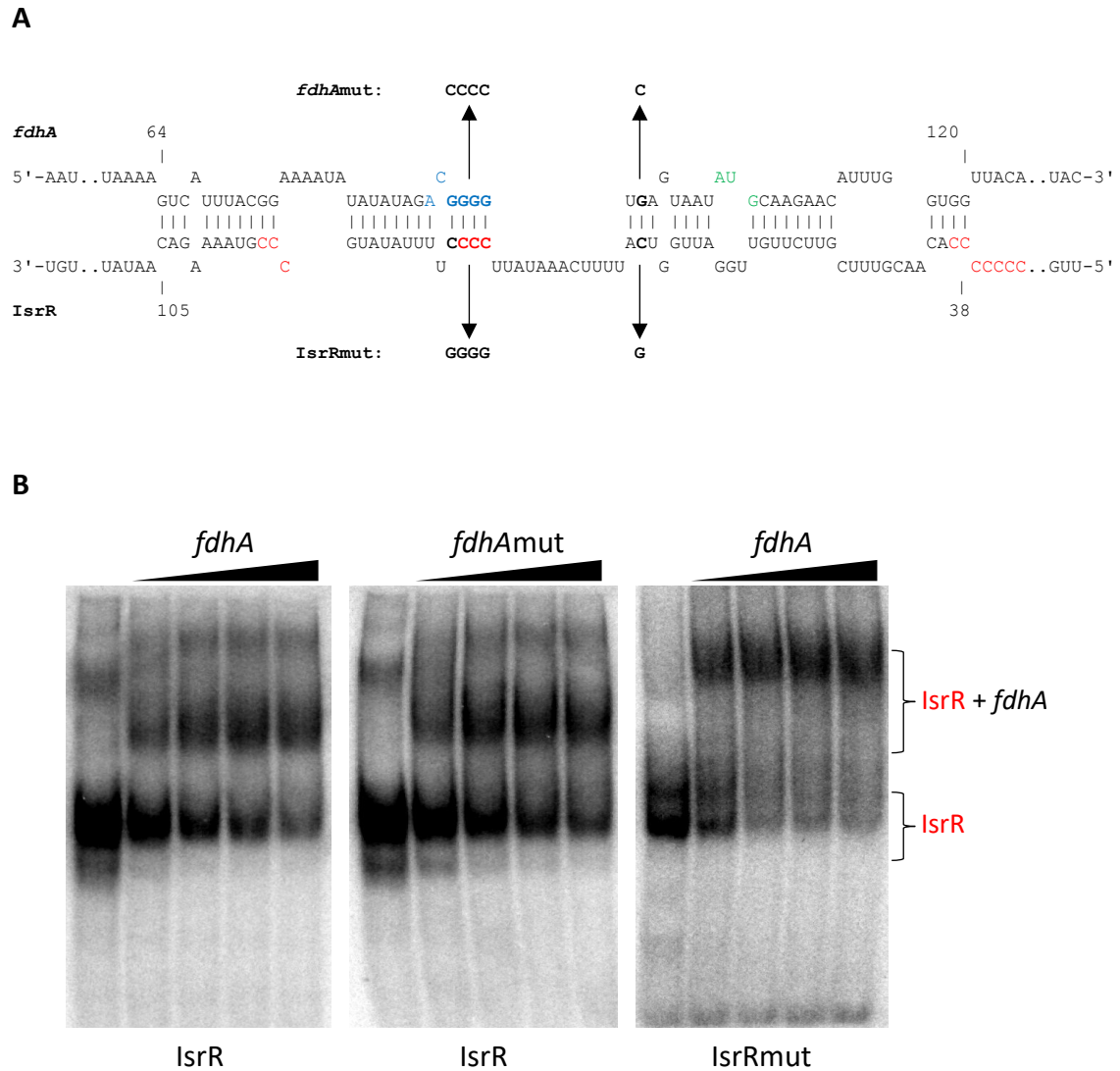
Adapted figure from a CopraRNA analysis with the 22 Staphylococci strains indicated using default parameters (31). Columns, investigated organisms; rows, targets; cell colors, IntaRNA p-value as indicated on the left panel; white cell, no homolog of a given target.

**Figure S6. IsrR putative targets involved in nitrate respiration pathway**



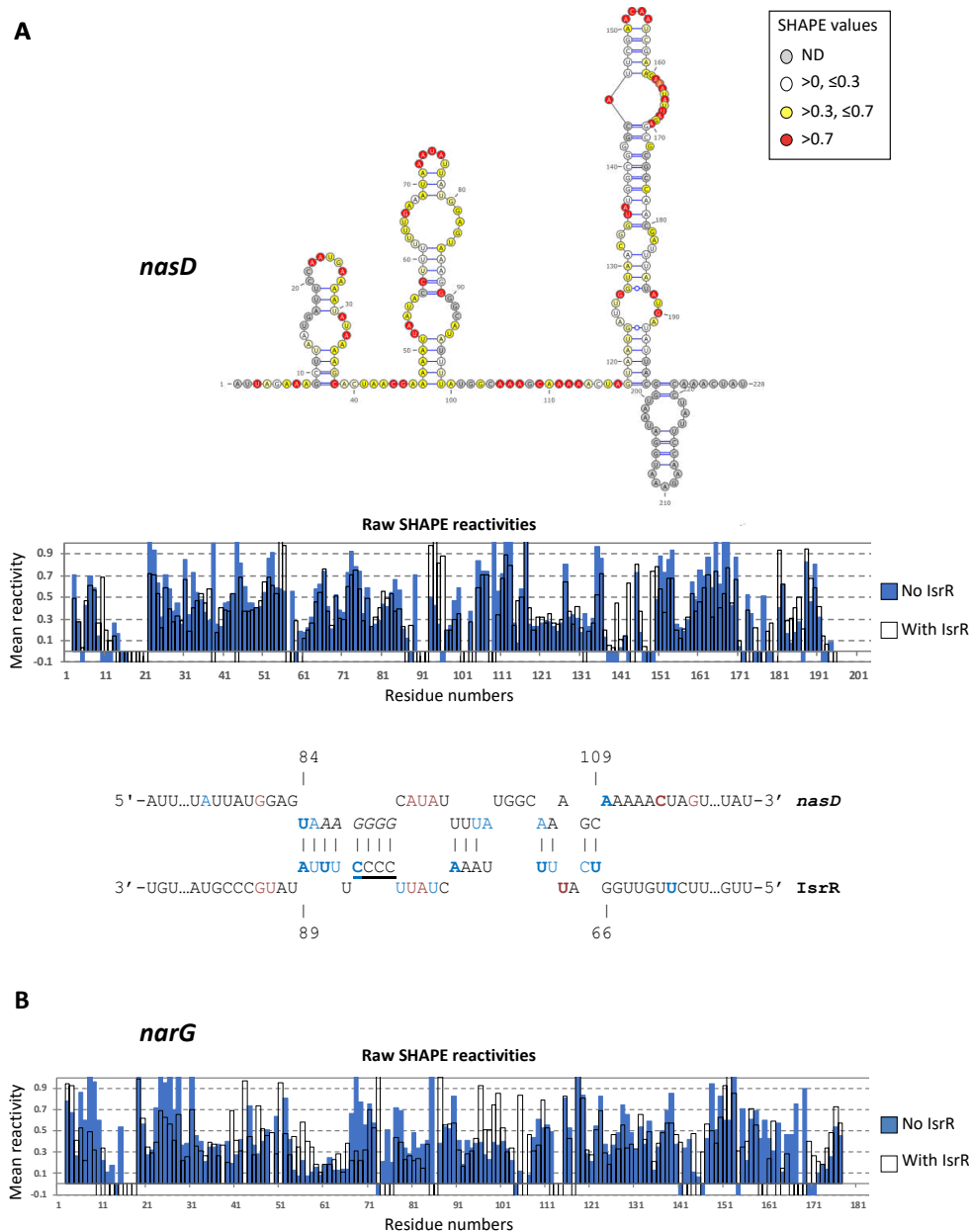
A) Dissimilatory nitrate reduction pathway. Adapted from BioCyc (32). B) Predicted interactions between IsrR and four putative targets (*fdhA*, *gltB2*, *narG* and *nasD* mRNAs) by IntaRNA. Input: IsrR complete sequence. For *fdhA*, *gltB2* and *narG*, sequence comprising reported +1 TSS and 100 nt downstream the start codon; for *nasD*, sequence comprising 100 nt upstream start codon and 100 nt downstream start codon. Green, start codon; blue, ribosome binding site; red, IsrR CRRs. Hybridization energy, interaction energy as determined by IntaRNA; Energy, hybridization energy minus energy to open RNA structures.

**Figure S7. Electrophoretic mobility shift assay of IsrR in the presence of *fdhA* mRNA**



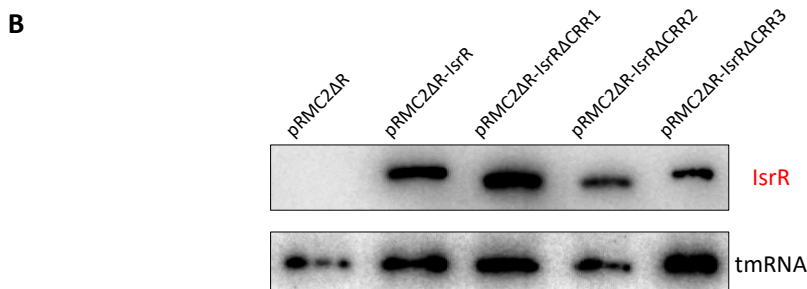
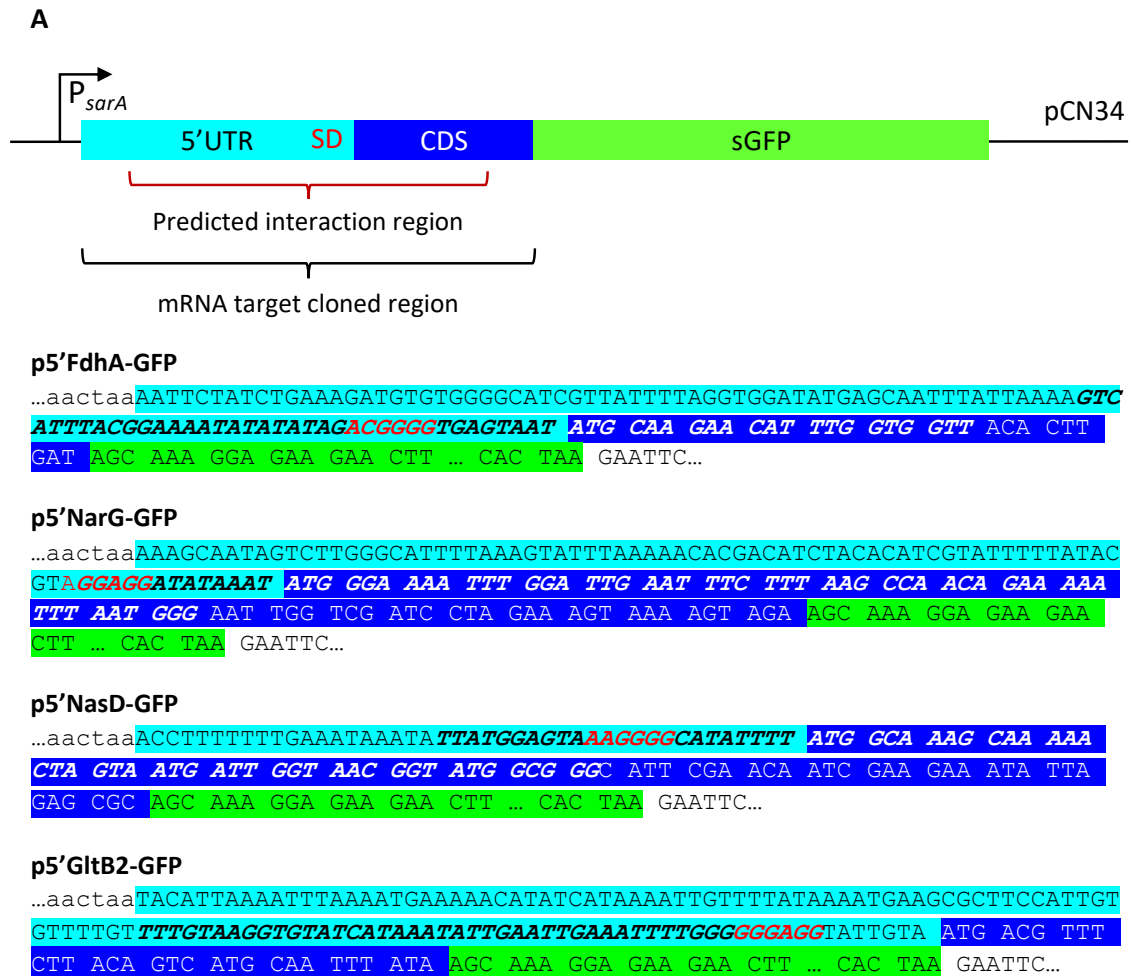
A) Predicted interaction between IsrR and *fdhA* mRNA as shown in Figure S5. Nucleotides mutated to potentially disrupt interaction are in bold. Inserted point mutations (G $\leftrightarrow$ C) are indicated with an arrow, resulting in IsrRmut and *fdhAmut*. B) Northern blot experiments with probe detecting IsrR. Left panel: constant amounts of IsrR alone (first column) or in the presence of increasing amounts of *fdhA* mRNA. Middle panel: constant amounts of IsrR alone (first column) or in the presence of increasing amounts of *fdhA* mRNA harboring five point mutations (*fdhAmut*). Right panel: constant amounts of IsrR harboring five point mutations (IsrRmut) alone (first column) or in the presence of increasing amounts of *fdhA* mRNA. In all cases, 5 pmol either of IsrR or IsrRmut were used. For *fdhA* or *fdhAmut*, 25, 50, 100 and 200 pmol were used.

**Figure S8. Comparison of *nasD* and *narG* mRNA reactivity obtained in presence or absence of IsrR with proposed alternative interaction model**



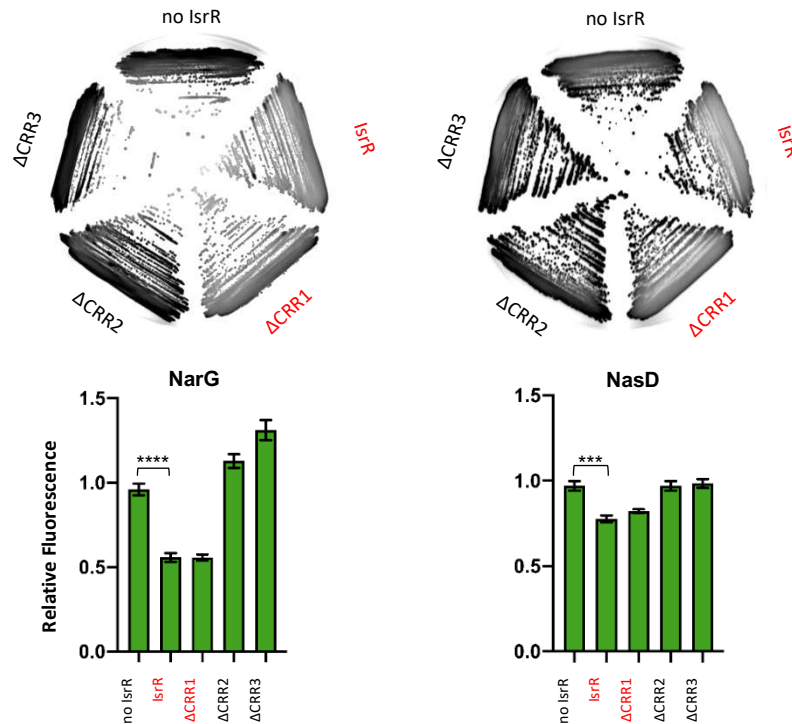
*nasD* (A) and *narG* (B) mRNAs were probed with 1M7 at 37°C in 40 mM HEPES pH 7.5, 150 mM KCl and 5 mM MgCl<sub>2</sub>. Top panels: Secondary structure of target RNA obtained with IPANEMAP using 1M7 reactivity as soft constraints. Nucleotides are colored according to their reactivity in the absence of IsrR. Of note, secondary structure of *narG* mRNA could not be obtained due to variable reactivity profile across the whole sequence. Middle panels: average reactivity of each nucleotide within the mRNA molecule in presence (white bars) or absence (blue bars) of IsrR. Bottom panels: proposed alternative model of interaction between target mRNA and IsrR considering the changes in reactivity in the presence of IsrR. Nucleotides in blue presented a decreased reactivity while those in brown presented increased reactivity. Nucleotides colored and in bold presented a significant change in reactivity in presence of IsrR (P value <0.05, n=3). mRNA Shine-Dalgarno sequence is shown in italics, IsrR CRR2 region is underlined.

Figure S9. Reporter fusions associated to nitrate respiration for *IsrR* activity tests



A) Upper part: Schematic representation of constructed reporter fusions. Below: *fdhA*, *narG*, *nasD* and *gltB2* cloned sequences. All sequences include the 5'UTR and first codons of *IsrR* target genes cloned in frame with the super-folder *gfp* CDS (minus its start codon). 5'UTR of *IsrR* target, light blue; first codons of *IsrR* target, dark blue; first and last sGFP codons, green; Shine-Dalgarno sequence, red; corresponding sequence to the predicted *IsrR* pairing region, bold italic font. B) *isrR* and its  $\Delta$ CRR derivatives expression. Northern blot experiment. Total RNA extracts from HG003  $\Delta$ *isrR* derivatives containing the indicated plasmids. Membranes were probed for *IsrR* and tmRNA (loading control).

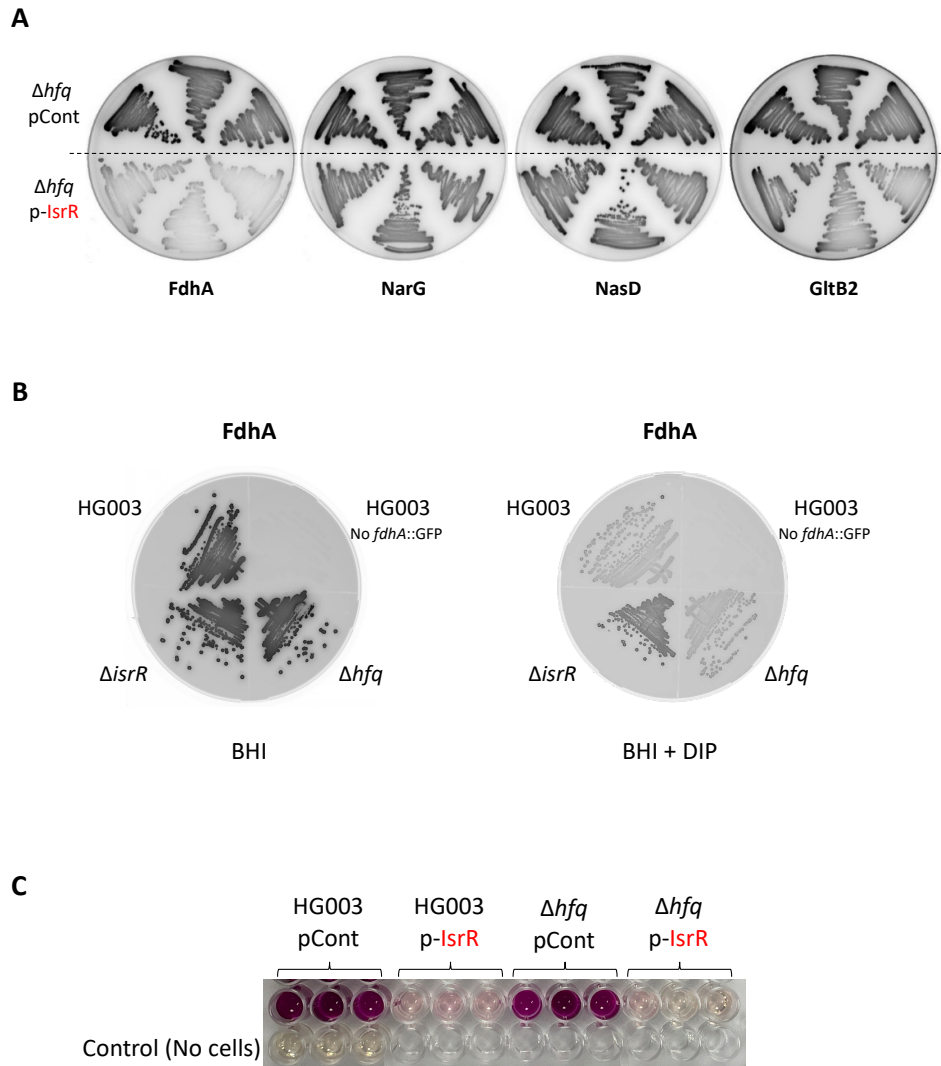
**Figure S10. Translational down-regulation by IsrR and CRR contribution**



Leader fusions between the first codons of *narG* and *nasD* with a GFP were constructed (Figure S9 and Table S2). The cloned fragments include the interaction regions with IsrR as described in Figure S6. HG003  $\Delta isrR$  derivatives with either a control plasmid (no IsrR; pRMC2 $\Delta R$ ), or plasmids expressing IsrR (pRMC2 $\Delta R$ -*isrR*), IsrR $\Delta CRR1$  (pRMC2 $\Delta R$ -*isrR* $\Delta CRR1$ ), IsrR $\Delta CRR2$  (pRMC2 $\Delta R$ -*isrR* $\Delta CRR2$ ), IsrR $\Delta CRR3$  (pRMC2 $\Delta R$ -*isrR* $\Delta CRR3$ ) were transformed with each engineered reporter gene fusions. Translational activity from the two reporters in the presence of the different *isrR* derivatives were evaluated by fluorescent scanning of streaked clones on plates (n=3). Fully active IsrR derivatives are shown in red. Translational activity of the reporter genes with the different *isrR* derivatives was also determined in liquid culture. The fluorescence of the 10 strains was measured in 6 h cultures using a microtiter plate reader. Results are normalized to 1 for each fusion with the control plasmid. Error bars indicate the standard deviation from three independent experiments (n=3). Statistical analysis was performed using Student t test; \*\*\*\* represents P value <0.0001, \*\*\* represents P value = 0.0006.



**Figure S11. Hfq is not required for IsrR activity**



A) Translational down-regulation of four targets by IsrR in  $\Delta hfq$  background. Leader fusions between *fdhA*, *narG*, *nasD* and *gltB2* are the same as in Figure 6 and S10. HG003  $\Delta hfq$  derivatives with either a control plasmid (pCont; pRMC2 $\Delta$ R), or a plasmid expressing IsrR (p-IsrR; pRMC2 $\Delta$ R-*isrR*) were transformed with either p5'*FdhA*-GFP, p5'*NarG*-GFP, p5'*NasD*-GFP or p5'*GltB2*-GFP. Translational activity from the four reporters in the presence of *isrR* or not, was evaluated by fluorescent scanning of streaked clones on plates (n=3). B) Translational down-regulation of *fdhA::GFP* leader fusion by IsrR. HG003 WT strain and its  $\Delta isrR$  and  $\Delta hfq$  derivatives were transformed with p5'*FdhA*-GFP and grown either in rich media (BHI), or BHI supplemented with DIP 0.5 mM. Expression of IsrR after iron chelation decreased translation of *fdhA* leader fusion in the  $\Delta hfq$  strain as observed with the WT strain. HG003 strain without leader fusion was included as control. C) Inhibition of nitrite production by IsrR. HG003 strain and its  $\Delta hfq$  derivative harboring a control plasmid (pCont; pRMC2 $\Delta$ R) or a plasmid expressing IsrR (p-IsrR; pRMC2 $\Delta$ R-*isrR*), were grown in rich media under anaerobic conditions. Nitrate (NaNO<sub>3</sub>) was added to the media and 150 min after, the nitrite produced was compared qualitatively using that Griess colorimetric method. Expression of IsrR inhibited nitrite production in the  $\Delta hfq$  strain as observed in HG003.

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## J. ADDITIONAL DISCUSSION

Through this work, the first example of a sRNA responsible to drive the iron-sparing response in *S. aureus* has been described. This sRNA, named here IsrR (iron-sparing response regulator), has proven to be a crucial regulatory element for the adaptation to low-iron conditions, regulation of nitrite production and virulence of this important human pathogen.

IsrR emerged from competition experiments using libraries of *S. aureus* sRNA-mutant strains grown under low-iron conditions. These assays have been under constant development and improvement for nearly 10 years, and they represent a reliable and broad strategy to find sRNA-related phenotypes (414–416). Since sRNAs are involved in “fine-tuning” regulatory mechanisms, and like their targets, may be expressed under very specific conditions, it is remarkably difficult to identify phenotypes related to sRNA-mutants. By generating independent libraries with tagged-mutants of all the reported *bona-fide* sRNAs (416), and subsequently growing them under stress conditions (*e.g.*, antibiotic exposure, pH and osmolarity variations, oxidative stress, nutrient deprivation), this strategy emulates the competition events that can occur when *S. aureus* infects the human host.

Since iron deprivation is one of the key strategies of the host nutritional immune response, the two competition experiments performed under low-iron conditions represent a valuable insight into bacterial sRNA-driven adaptation during infection. The fact that exclusively the  $\Delta$ *isrR* mutant showed a 10- and 1000-fold decrease when using the iron-chelators DIP and EDDHA, respectively (pg. 97), highlights the severe adaptative disadvantage of this strain in comparison with the other 48 mutants, and represents a remarkable phenotype caused by a single sRNA molecule. Spot tests comparing growth of the WT versus the  $\Delta$ *isrR* mutant under low-iron conditions produced similar results (pg. 97 and 147). Although the growth differences in these plates after around 16 hours growth are not visually striking, they show the onset of the growth defect caused by *isrR* inactivation, a detrimental effect that is visible to a greater extent after the 3-day growth during the competition assays.

Although this is the first study presenting a detailed description of IsrR regulation, it is not the first to feature this sRNA. In 2016, two studies presented interesting results regarding IsrR. First, Carrol *et al.* (371) showed that this sRNA, under the name of Tsr25, was the most highly upregulated sRNA when *S. aureus* was grown in human serum (583.02-fold change) in

comparison to rich media (TSB) after performing RNA-Seq analysis. This result was also confirmed by Northern blot experiments. However, no further discussion about Tsr25 was presented. When correlating these observations with our own results, IsrR (Tsr25) high expression in human serum is consistent since this blood component is deficient in iron, promoting IsrR expression after Fur de-repression.

On the other hand, Mäder *et al.* (370) proposed that IsrR, under the name of S596, was a *trans*-encoded sRNA potentially Fur-regulated. Northern blots showed increased levels of S596 in low-iron media (RPMI) when comparing to TSB. Additionally, a search for S596 putative targets using CopraRNA revealed that mRNAs encoding iron-sulfur cluster containing proteins (*e.g.*, *citB*, *fdhA*, *miaB* mRNAs), heme biosynthesis/containing enzymes (*e.g.*, *ctaA*, *hemE*, and *kata* mRNAs) and TCA cycle enzymes (*e.g.*, *citZ* and *sdhCA* mRNAs), could be regulated by this sRNA. However, no evidence was presented regarding these predicted regulations. As observed with human serum, increased expression of IsrR (S596) in RPMI is coherent with our results when using DIP to chelate iron from rich media. Also, several predicted targets in this study match our own *in silico* predictions. However, in Mäder *et al.*, CopraRNA analysis was limited to 8 *Staphylococcus* species, while our analysis used IsrR homologs from 22 *Staphylococcus* species, resulting in a broader search for conservation of IsrR-mRNA target interactions.

The role of IsrR upon iron adaptation was further analyzed by studying its regulation by Fur. Two Fur-binding motifs were found in the promoter region of *isrR*, the first one at position -34, and the second one starting just one nucleotide after *isrR* TSS. The presence of two Fur motifs is not unusual. According to the RegPrecise database (417), in *S. aureus* strain N315, the operons *isdA*, *isdCDEF-srtB-isdG*, *arlSR*, *sirCBA* and *sbnABCDEFGHI* have two Fur motifs in their promoter regions, while *ftnA* has three. All these genes (except for *arlSR*) encode proteins related to iron homeostasis. Considering that IsrR is the main effector of the iron-sparing response in *S. aureus*, it is reasonable to expect a tight and efficient regulation by Fur. Moreover, it was observed that an overexpression of IsrR under low-iron conditions is detrimental for *S. aureus* (pg. 97), which can be a consequence of the disruption of iron homeostasis. Hence the importance of assuring an effective Fur repression of *isrR* in order to maintain adequate transcript levels in accordance with the available intracellular iron.

IsrR secondary structure predicted by LocARNA (418) using the 22 IsrR homologous sequences from different staphylococcal species (pg. 148), shows that the nucleotide sequence and base-pair interactions of stems H1 and H2 are highly conserved among staphylococci, whereas H3 and

the terminator are more variable. These observations made us speculate that stems H1 and H2 might interact with a so far unknown RNA-binding protein different from Hfq. This hypothesis will be explored by our team to search for a putative IsrR-chaperone protein that could be important for IsrR regulations. Interestingly, the predicted secondary structure also showed that IsrR has three conserved C-rich regions (CRRs), probably exposed. These CRRs are likely important for IsrR activity since they potentially bind to G-rich mRNA target RBS.

A first experimental attempt to elucidate IsrR secondary structure *in vitro* was done using SHAPE (pg. 149). The proposed IsrR structure considered both, thermodynamic stability of intramolecular base-pairs, and individual nucleotide reactivity to 1-Methyl-7-nitroisatoic anhydride (1M7), and it is identical to LocARNA predicted model. Due to premature stops during T7 transcription of the 3' end of IsrR, the last 14 nucleotides could not be analyzed by SHAPE. Most nucleotides with high reactivity were identified in predicted single-stranded regions, however, several nucleotides that were predicted to be part of base-pair interactions also presented high reactivities (*e.g.*, nucleotides in stems H1 and H2). This could be explained by the formation of alternative secondary structures during 1M7 treatment, generating alternative single-stranded regions with high reactivity. Unfortunately, it was not possible to confirm that the CRRs are exposed since these regions presented low SHAPE reactivities. Therefore, improvements in this methodology and other structural analyses will be performed to either confirm the SHAPE reactivity results or to obtain an alternative IsrR secondary structure with CRR accessibility.

In many cases, the interaction of an sRNA with its mRNA target results in degradation of both transcripts after RNase recruitment. However, IsrR expression by growth with dipyriddy apparently did not affect the half-life of *fdhA* and *gltB2* mRNAs after rifampicin addition (pg. 105). However, since addition of rifampicin rapidly inhibits transcription of both IsrR and target mRNAs, an accurate description of IsrR-dependent mRNA degradation is difficult to achieve. Additionally, IsrR remarkable long half-life may be due to the inhibition of target mRNAs transcription after rifampicin addition, and consequent prevention of degradation of mRNA-bound IsrR by nucleases such as RNase III. In other words, absence of mRNA targets may increase IsrR stability. Of note, for the cultures grown in low-iron conditions, dipyriddy was added to the media since the start of the incubation. By the time the culture reached OD<sub>600</sub> 1.5 (≈5 hours), the stability of both mRNAs might be affected after prolonged growth under iron-deprivation. In order to overcome these issues, two strategies described by Massé *et al.* regarding RyhB-dependent degradation of *sodB* mRNA (419), could be extrapolated to IsrR regulation: i) Cells

are grown in rich media, IsrR synthesis is induced with an iron chelator, and some minutes after, an excess of iron is added to inhibit IsrR production. ii) IsrR expression is controlled by an inducible promoter, which could avoid interferences due to iron and Fur effects. In both cases, a controlled, short induction of IsrR would be achieved. These strategies could result in a more accurate description of IsrR-dependent regulation upon its mRNA targets. Additionally, the use of RNase mutant strains would provide more information about the putative role of the different elements of the *S. aureus* degradosome involved in IsrR regulation.

To further study the interaction between IsrR and its mRNA targets, EMSA was performed with IsrR and *fdhA* mRNA. A band-shift corresponding to the formation of a IsrR/*fdhA* mRNA complex was observed. However, after inserting five point mutations in one of the different predicted interaction regions of each one of the two RNA molecules, including the CRR2 of IsrR and the RBS of *fdhA* mRNA, the sRNA/mRNA complex was not disrupted (pg. 152). The interaction between IsrR and *fdhA* mRNA is predicted to be impressively long (41 base-pairs) and strong (hybridization energy of -39.36 kcal/mol). Therefore, it is possible that five point mutations might not be enough to efficiently disrupt the RNA duplex, since other regions can still form stable bindings (e.g., IsrR CRR1 or CRR3). These results, although suggesting IsrR/*fdhA* mRNA direct interaction, put in evidence the fact that some approaches that are widely used in other bacteria (i.e., complementation by site-directed mutagenesis) are not always feasible in bacteria such as *S. aureus*.

As an additional approach to study the interaction between IsrR and *fdhA*, *gltB2* and *nasD* mRNAs, SHAPE technology was employed (pg. 107 and 153). The robustness and reliability of this technique was demonstrated after analyzing the secondary structure of *fdhA*, *gltB2* and *nasD* mRNAs *in vitro*. In the three cases, a high correlation between the thermodynamic stability of intramolecular base-pairs and individual nucleotide reactivity to IM7 was observed, i.e., nucleotides predicted to be in single-stranded regions presented a high reactivity. Of note, *narG* secondary structure could not be obtained since most of its nucleotides presented high reactivities, which indicates that when this mRNA when alone, it is not stable and forms diverse secondary conformations in the tested conditions. However, important changes in its reactivity were observed in the presence of IsrR, presumably after a direct interaction. For the other three IsrR targets, the observed changes in reactivity of these mRNAs when exposed to IsrR, demonstrate the modification of their secondary structure after base-pairing with IsrR, and particularly, with IsrR CRR2. Interestingly, according to SHAPE models of interaction, IsrR CRR2 directly binds to the RBS of *fdhA*, *gltB2* and *nasD* mRNAs, strongly suggesting an effect of IsrR



upon the translation of these mRNA targets. In overall, SHAPE results confirm direct interaction between IsrR and *fdhA*, *gltB2*, *nasD*, and possibly *narG*, mRNA targets with a consequent posttranscriptional effect, demonstrated with the GFP reporter fusions.

Employing translational reporter fusions, a first experimental approach was performed to describe IsrR regulation upon translation of *fdhA*, *gltB2*, *narG*, and *nasD* mRNAs. In all cases, over-expression of IsrR significantly decreased mRNA::*gfp* translation (pg. 109 and 155). Although there was no alteration of *fdhA* and *gltB2* mRNAs half-life due to IsrR, repression of both mRNAs translation can be enough for an efficient IsrR-downregulation mechanism. As described by Morita *et al.*, sRNA translational repression is sufficient for gene silencing. Whereas sRNA-mediated degradation of target mRNAs may serve as an additional mechanism to eliminate translationally inactive mRNAs, making gene silencing irreversible (420).

Furthermore, the role of IsrR CRRs (C-rich regions) was investigated. When comparing to full-length IsrR, deletion of the different CRRs resulted in important changes of mRNA::*gfp* translation rates, with specific CRRs being necessary for regulation of a particular target: CRR1 and CRR2 were necessary for translation inhibition of *fdhA*::*gfp* and *gltB2*::*gfp* (pg. 109), while CRR2 and CRR3 were necessary for translation inhibition of *narG*::*gfp* and *nasD*::*gfp* (pg. 155). Of note, CRR2 was not predicted to be involved in the interaction between IsrR and *gltB2* according to *in silico* predictions. However, after deletion of this motif, an important restoration of *GltB2*::GFP expression was observed. This may be due to alternative IsrR-*gltB2* mRNA interactions not shown in the top *in silico* predictions, where CRR2 can bind to *gltB2* mRNA and alter its translation. Furthermore, the decrease in fluorescence of *NasD*::GFP after IsrR expression, although significant, was moderate in comparison with the other three targets. This can be explained by the remarkably strong fluorescence of the p5'*NasD*-GFP construction, which may hinder IsrR downregulation effects. In overall, these results show the importance of the three CRRs for IsrR activity, especially CRR2, which seems necessary for downregulation of the four targets presented in this study. These observations are supported by SHAPE data. However, additional experiments introducing point mutations within IsrR CRRs are necessary, since although short, deletions in IsrR sequence might result in destabilization of the sRNA molecule.

Finally, two impressive phenotypes highlight the importance of IsrR within *S. aureus* metabolism adaptation and virulence. i) Complete inhibition of nitrite production after IsrR accumulation *in vivo* (pg. 110). By inhibiting the translation of the most important enzymes of nitrate

metabolism, *i.e.*, nitrate and nitrite reductases *narG* and *nasD*, respectively and putatively the nitrate response regulator *nreC* (see below, pg. 168), IsrR tightly represses this pathway and promotes iron economy by preventing the production of iron-using proteins. There are several examples of bacterial sRNAs regulating nitrate metabolism. In *E. coli*, SdsN<sub>137</sub> and RprA sRNAs negatively regulate the synthesis of NarP, a nitrate/nitrite responsive transcriptional regulator (421,422); while RyhB represses the expression of the nitrite reductase *nirB*, and possibly *narP* (205,421). However, in these studies, no sRNA-dependent phenotypes *in vivo* were presented. To gain further insight into IsrR regulation upon nitrate metabolism, additional tests such as quantification of different nitrate metabolites and protein analysis by western blot, may be resourceful. ii) IsrR is required for *S. aureus* full virulence. In a murine model, inoculation of the WT and  $\Delta$ *isrR*-complemented strains resulted in death of most mice after 8 days, while a  $\Delta$ *isrR* mutant strain significantly decreased *S. aureus* lethality (pg. 110). These results establish the crucial role of IsrR during *S. aureus* infection. Together with other sRNAs involved in *S. aureus* virulence such as RNAIII, Teg41, Teg49, SprD and SprX (143,279,389,410,423,424), IsrR demonstrates the importance of sRNAs in bacterial pathogenesis.

## K. CONCLUSIONS AND PERSPECTIVES

IsrR is the first example of a sRNA dictating the iron-sparing response in the human pathogen *S. aureus*.

During bacterial infection, the host elicits the nutritional immune response and efficiently sequesters essential nutrients such as iron to prevent bacterial proliferation. However, bacteria have developed numerous mechanisms to adapt and thrive in these iron-deprived conditions. One of these mechanisms is the iron-sparing response, which is driven by sRNAs in different bacteria, such as RyhB in *E. coli*, PrrF in *P. aeruginosa*, NrrF in *N. meningitidis*, FsrA and *B. subtilis*, and MsrI in *M. tuberculosis*. However, until now, this response had not been elucidated in *S. aureus*.

Through the results presented in this study, IsrR was found necessary for *S. aureus* optimal adaptation and survival under low-iron conditions. After being expressed in a tightly iron-dependent manner, IsrR inhibits the expression of non-essential proteins using iron, so this element can be re-directed to vital cellular processes. By this mechanism, IsrR efficiently ensures iron-economy within the cell, and allows *S. aureus* to overcome nutrient limitations imposed during infection.

IsrR shares several targets and a similar regulatory mechanism with the aforementioned sRNAs. However, their sequence, conformation and RNA chaperone-dependency are distinct. Therefore, we concluded that these sRNAs are functional analogs that depict how through evolution, RNA-based regulation represents an optimal solution for bacterial adaptation to iron deprivation.

Considering its broad conservation among *Staphylococcus* species and its important role in *S. aureus* pathogenesis, this study reveals IsrR as a potential new target for therapeutic strategies against staphylococcal infections, which represent an important health burden worldwide.

Several features about IsrR regulation remain to be explored. In-depth study of its secondary structure and possible interaction with an RNA-chaperone protein, will surely unveil more details about IsrR mode of action. So far, IsrR regulation upon targets involved in nitrate metabolism has been described, however, regulation of other putative targets involved in other

important cellular processes such as citrate metabolism (*e.g.*, *citB*, *citM* and *ccpE* mRNAs), cell division (*e.g.*, *mraZ* mRNA) and oxidative stress-response (*e.g.*, *msrA* and *katA* mRNAs), among others, will be addressed. A detailed study of these regulations might reveal new IsrR mechanisms of action and describe the contribution of this global regulator in diverse physiological processes within *S. aureus*, thus, opening the way to different projects aiming to further demonstrate the crucial role of sRNAs in bacterial adaptation and pathogenesis.

## L. ADDITIONAL STUDIES

### 1. Study of IsrR regulation upon the nitrate metabolism regulator, NreC

*nreC* mRNA, encoding for the nitrate response regulator, is within the best 20 IsrR putative targets obtained by CopraRNA (pg. 133). This target is likely relevant since among the best candidates are *fdhA*, *narG*, *nasD* and *gltB2* mRNAs all encoding proteins involved in the nitrate metabolism in *S. aureus*, and for which IsrR inhibits their translation (pg. 95 and 137). The subsequent discussion will focus on the relationship between IsrR and *nreC*.

In *S. aureus* NCTC8325 strain, NreC is part of the *nreABC* operon (Fig. 31). This complex acts as the transcriptional activator of the *nar* and *nas* operons, which encode the nitrate and nitrite reductases, respectively, as well as its own operon *nre*. NreB is a histidine kinase sensor active only under anoxic conditions and after conformational changes triggered by nitrate-bound NreA. It phosphorylates NreC, a DNA-binding response regulator, which in turn binds to specific motifs upstream the aforementioned operons and induces their transcription (Fig. 32).

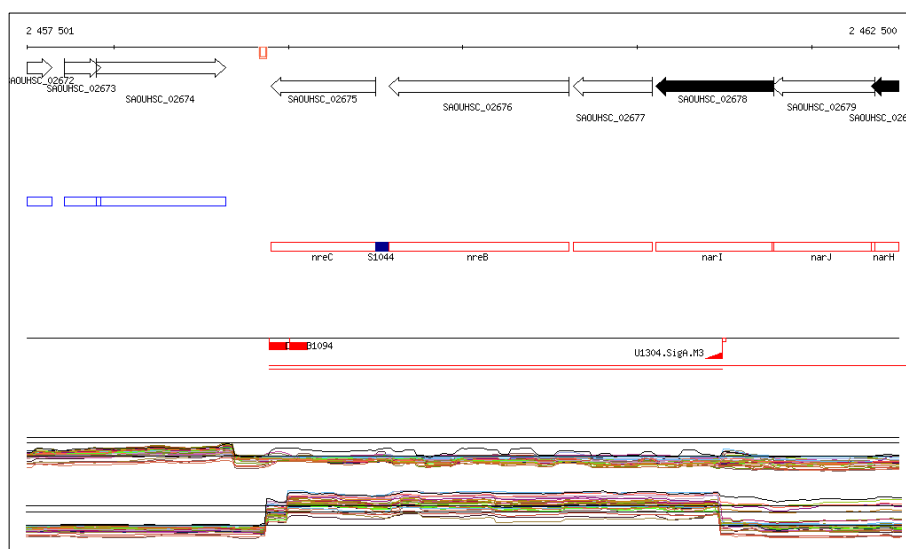


Figure 35. Genomic location and expression profile of *nreABC* operon.

Upper panel shows GenBank annotation; Middle panel (blue and red rectangles) shows proposed annotation of transcription segments; Middle panel (red lines) shows transcription units with promoters as triangular flags and terminators as rectangular flags; Lower panel shows expression profile of WT *S. aureus* HG001 in 30 representative growth conditions (370). *nreA* gene (SAOUHSC\_02677) is not annotated since it was until recently that its nitrate-responsive function was elucidated (272,273). Taken from *S. aureus* Expression Data Browser (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>) (370).

While NreC does not contain a Fe-S cluster, NreB does (270), as well as proteins encoded by its regulon (*i.e.*, *narG* and *nasD*) (267). The proposed function of IsrR is to down-regulate the amount of iron containing enzymes. We demonstrated that the accumulation of IsrR prevents the translation of operon expressing iron-containing proteins. Here, we hypothesized that under anoxic and low-iron conditions, IsrR would also prevent the expression of the nitrate respiration transcriptional activator.

This hypothesis highlights a peculiar mechanism of regulation by a sRNA, in which IsrR would have an effect on both, the transcriptional activator NreC, as well as the genes regulated by this protein (*i.e.*, *nas*, *nar* and *nre* operons). This would result in a dual regulation that would assure a tight control of this pathway at different steps and in consequence promote overall iron economy within the cell (Fig. 32). A similar regulatory mechanism can be found in *E. coli*, where SdsN137 and RprA sRNAs negatively regulate the synthesis of NarP, a nitrate/nitrite responsive transcriptional regulator (8,9); while RyhB represses the expression of the nitrite reductase *nirB*, and possibly *narP* (8,10).

We therefore question the possible role of IsrR upon *nreC* expression.

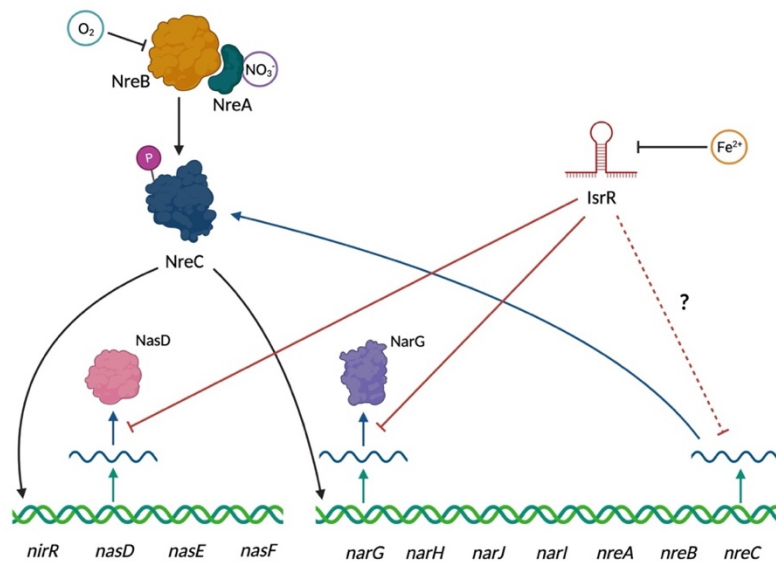


Figure 36. IsrR regulation of *nreC*, *narG* and *nasD*.

In the absence of oxygen and in presence of nitrate, phosphorylated NreC activates transcription of *nas*, *nar* and *nre* operons. Under low iron conditions, IsrR would be expressed and probably able to regulate the expression of NreC and therefore indirectly altering transcription of *nas*, *nar* and *nre* operons. Additionally, it has been shown that IsrR inhibits translation of iron-containing proteins NasD and NarG (pg. 137), assuring a tight control in the overall expression of proteins within the nitrate respiration pathway.

## 1.1. Effect of IsrR upon *nreC* mRNA levels

The effect of IsrR upon *nreC* mRNA was studied by monitoring the amount of *nreC* mRNA by Northern blots in strains with different levels of IsrR. We first compared a  $\Delta fur$  mutant and a  $\Delta fur\Delta isrR$  double mutant; among these, the strain  $\Delta fur$ , leading to a constitutive expression of IsrR, showed a significant increase in the levels of *nreC* mRNA in its polycistronic and processed forms (Fig. 33A). Next, to prevent possible undesired effects generated by the absence of Fur, a second set of strains consisted of a  $\Delta isrR$  mutant carrying either an empty plasmid or a plasmid over-expressing IsrR. Strains were grown under anaerobic conditions in the presence of nitrate ( $\text{NaNO}_3$ ). The strain expressing IsrR showed significantly higher levels of *nreC* mRNA compared to the isogenic strain with the control plasmid (Fig. 33B).

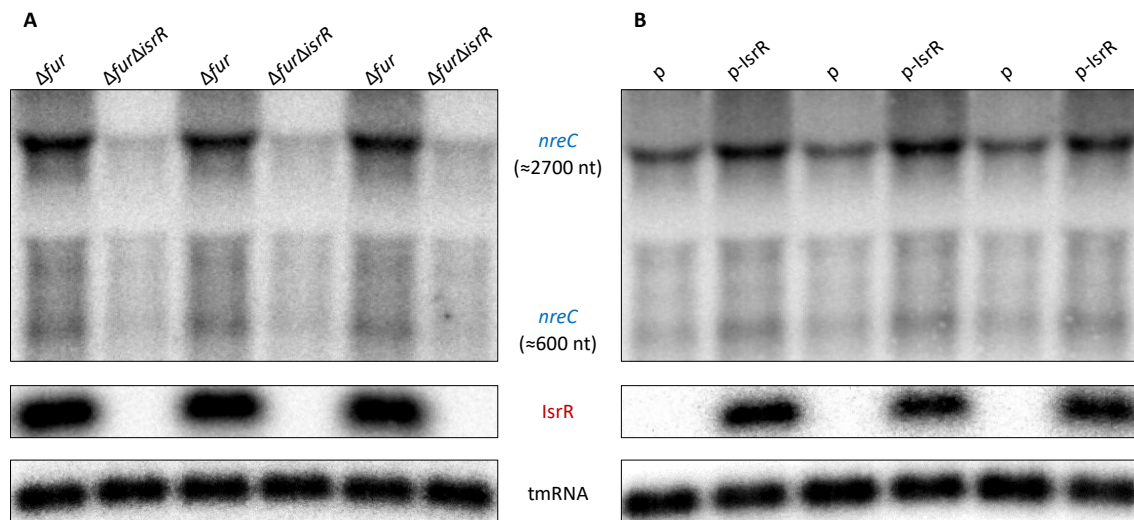


Figure 37. Upregulation of *nreC* mRNA by IsrR.

A) Northern blot showing levels of *nreC* in strains with a constitutive expression of IsrR ( $\Delta fur$ ) or without IsrR ( $\Delta fur\Delta isrR$ ); grown in rich media until  $\text{OD}_{600}$  1.5. B) Northern blot showing levels of *nreC* in strains over-expressing IsrR (p-IsrR) or without IsrR (p, control plasmid); grown in anaerobiosis and in the presence of nitrate. For both membranes, expression of IsrR is shown, as well as tmRNA, which is used as a loading control.

These first results show that IsrR leads to an increased amount of *nreC* mRNA suggesting that IsrR acts “positively” on *nreC* mRNA, possibly at the post-transcriptional level. This unexpected observation is reminiscent of observation described thereafter.

*Salmonella enterica* is a facultative anaerobic bacterium from the Enterobacteriaceae, which can use nitrate as a final electron acceptor in a similar way to *S. aureus*. In *S. enterica*, NarL serves as a transcriptional activator of the nitrate and nitrite reductases (comparable to NreC) and

additionally, *S. enterica* possesses two RyhB homologs named RyhB1 and RyhB2, which in some studies are named RfrA and RfrB, respectively. Under aerobic conditions, the expression of RyhB after the addition of an iron chelator or the use of a *fur* mutant, increases the levels of *narL* mRNA, whereas under anaerobic conditions, these levels are also increased but in a lesser degree due to the partial repression upon *narL* by the Fnr regulator, which is active only in the absence of oxygen. Moreover, in *S. enterica*, a  $\Delta rfrA\Delta rfrB$  double mutant presented decreased levels of *narL* mRNA (425).

Despite no sequence similarities between NarL and NreC, nor between RyhB1/2 (RfrA/B) and IsrR, the iron-responsive sRNAs of both bacterial species share putative similar targets. RyhB and IsrR are likely functional analogs.

These preliminary results need to be supported by complementary experiments addressing the nature of the IsrR/*nreC* mRNA interaction and the stability of *nreC* over time (*i.e.*, mRNA half-life).

## 1.2. *In silico* analysis of IsrR-*nreC* interaction

*In silico* analysis predicted interaction between IsrR sRNA and *nreC* mRNA (Fig. 34).

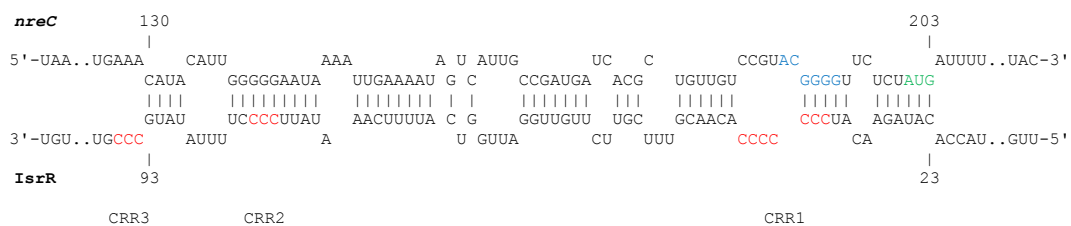


Figure 38. IsrR and *nreC* predicted interaction by IntaRNA.

IsrR C-rich regions (CRR1 to 3) are highlighted in red; *nreC* ribosome binding site and start codon are highlighted in blue and green, respectively.

The predicted pairing presents features seen previously with other putative targets of IsrR (pg. 134). It consists of a long (50 base-pairs) and stable interaction (hybridization energy of -48.19 kcal/mol). Two out of three IsrR C-rich regions (CRRs) are involved in this interaction: 1) CRR1 binds partially with the RBS of *nreC* mRNA and 2) CRR2 interacts upstream of this site. CRR3 seems not to participate in the pairing with *nreC* mRNA. This prediction suggests that IsrR could act at the post-transcriptional level by impeding translation of *nreC* mRNA after binding to its



RBS and start codon. In this way, production of NreC would be inhibited and subsequent transcription of *nar* and *nas* operons, prevented.

To confirm this prediction, an *in vivo* reporter assay was constructed and tested as described (pg. 79).

### 1.3. NreC-GFP reporter assay

A plasmid expressing IsrR, and a second one containing the 5'UTR and the first ten codons of *nreC* in frame with *gfp* were transformed into *S. aureus* RN4220. Additionally, a strain expressing the sRNA RsaH (which presumably has no effect upon *nreC*) instead of IsrR, was used as control. Both strains were grown in liquid rich media and after 6 hours, the OD<sub>600</sub> and fluorescence were measured (Fig. 35). A significantly decreased fluorescence in the strain expressing IsrR was observed.

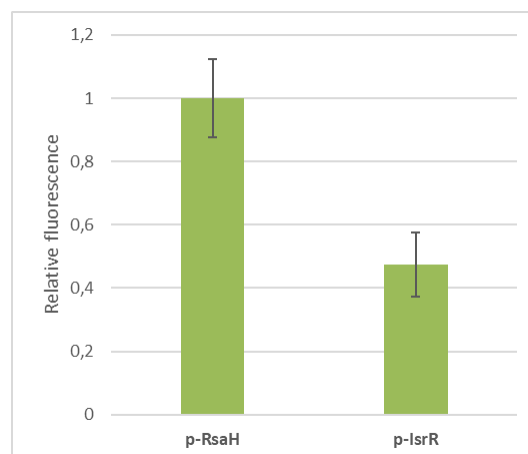


Figure 39. Inhibition of translation of *nreC::gfp* by IsrR.

Results of the two-plasmid reporter system, with *S. aureus* cells harboring the plasmid with the translational fusion *nreC::gfp*, containing a second plasmid expressing either RsaH (sRNA used as control) or IsrR. After 6h growth, fluorescence was measured and normalized to the respective OD<sub>600</sub>. N=3, *p*-value=0.005061.

This experiment suggests that IsrR inhibits the translation of *nreC::gfp*, most probably by binding to *nreC* RBS and therefore blocking the entry of the ribosome. While this regulation would be in accordance with above shown *in silico* predictions, additional tests such as point-mutagenesis are necessary to determine which regions of IsrR are responsible for this down-regulation.

In overall, these results show that IsrR increases *nreC* mRNA levels, while *in silico* analysis suggests the binding of IsrR to *nreC* RBS would inhibit its translation. This hypothesis is supported with the GFP reporter system, which indeed showed a decrease of *nreC* translation in the presence of IsrR.

Although this kind of regulatory mechanism is not usual in enteric bacteria, we suggest that it could be more frequent within the Firmicutes phylum. This can be explained as follows: under iron starvation conditions, *S. aureus* would express IsrR to stabilize the mRNA of NreC. By inhibiting *nreC* translation (as it does with *narG* and *nasD*), it tightly controls their expression and spare essential iron. When this element is restored, high levels of *nreC* mRNA would be readily available to restart nitrate metabolism, a process more energetically efficient than fermentation (Fig. 36).

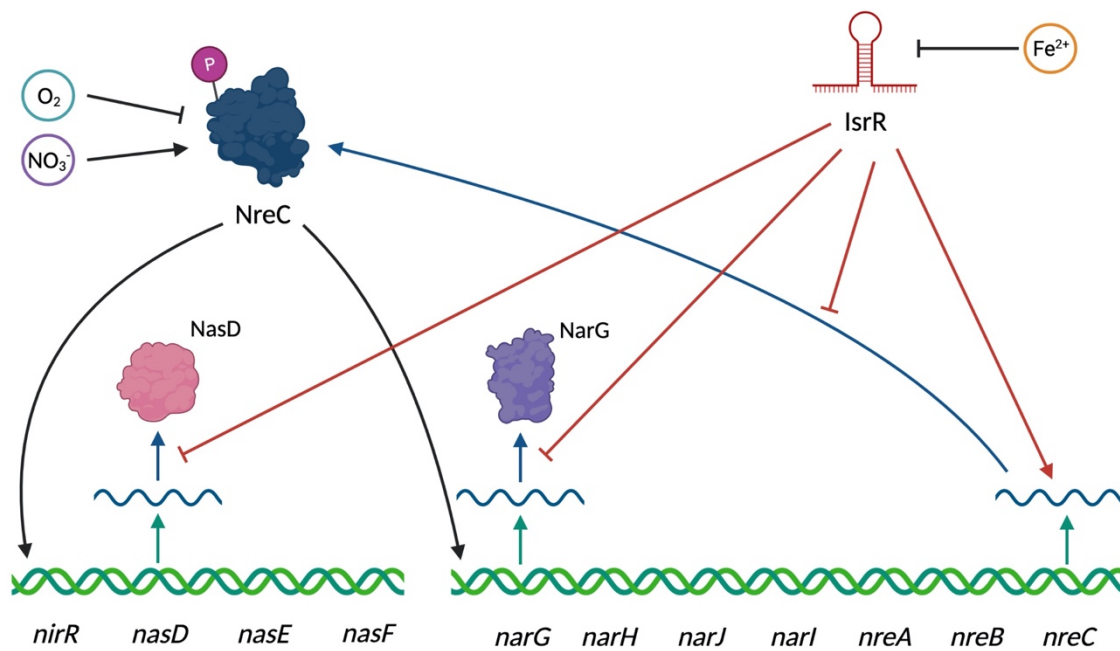


Figure 40. Proposed model for IsrR regulation upon *nreC*.

When IsrR is expressed in low iron conditions, it increases the levels of *nreC* mRNA (probably due to stabilization after binding), and possibly generating a pool of *nreC* mRNAs. Nevertheless, IsrR binding inhibits translation of *nreC* after blocking ribosome entry. Once iron levels are restored, IsrR would not be present and readily available *nreC* mRNAs would allow a rapid generation of NreC regulator and subsequent reactivation of nitrate metabolism.

Finally, there are numerous details still needed to elucidate regarding the interaction of IsrR and *nreC*, nevertheless, these studies show an example of a dual regulation by an iron-responsive

RNA upon a regulator of nitrate metabolism within *S. aureus*; and thus, highlighting the important role of sRNAs in numerous cellular processes within pathogenic bacteria.

## 1.4. Methods

**Strains and plasmids.** For *S. aureus* strains and plasmids used, see pg. 108 and 113 .

**Northern blots.** For culture growth conditions, RNA extraction and Northern blotting, see pg. 77. Probes for *IsrR*, *nreC* mRNA and tmRNA detection presented in pg. 124.

***In silico* prediction of *IsrR-nreC* interaction.** IntaRNA 2.0 (426) was used to predict interactions between *IsrR* and *nreC* RNAs. *nreC* sequence from *S. aureus* NCTC 8325 comprising -200 and +100 nucleotides from the start codon was considered as input. Output parameters: suboptimal interactions can overlap in both; lonely base pairs and GU at helix ends accepted. Seed parameters: minimum 7 base-pairs in seed; seeds with GU base pairs and GU ends accepted.

**NreC-GFP reporter assay.** As with the plasmid over-expressing *IsrR* (pg. 113), control plasmid over-expressing *RsaH* was constructed by cloning full *rsaH* gene under the control of the constitutive P<sub>Tet</sub> promoter of plasmid pRMC2 after deleting its Tet repressor (pRMC2ΔR). For the NreC-GFP reporter plasmid, the region comprising 20 nucleotides upstream the first nucleotide predicted to interact with *IsrR*, and 30 nucleotides after *nreC* start codon (*i.e.*, 10 first codons), was cloned in frame with a *gfp* coding sequence deprived of its ATG into the multicopy plasmid pCN34.

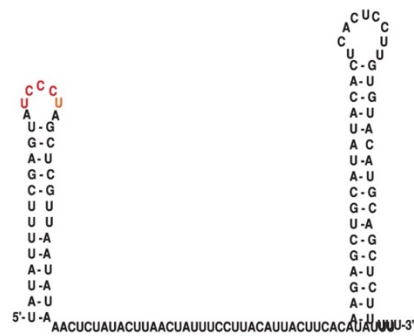
## 2. Study of *RsaX20* putative sRNA involved in metal homeostasis in *S. aureus*

*RsaX20* is a putative sRNA conserved among different staphylococcal species first described by Geissmann *et al.* in 2009 in *S. aureus* as a transcript of 116 nt with a C-rich motif (177). Its corresponding coding gene, *rsaX20* ends with a Rho-independent terminator and is located between the *SA2203* and *SA2204* genes (N325 strain nomenclature). For these reasons, *RsaX20* was proposed to be a sRNA. However, the same study suggested that *RsaX20* had an upstream putative small ORF encoding a hypothetical protein (annotated *SAOUHSC\_02702* in strain

NCTC8325). RsaX20 was subsequently also reported in studies on sRNAs with distinct names such as *srn\_4520* (369), *S1052* (370) and *Teg130* (364).

In 2010, *Beaume et al.* proposed a RsaX20 secondary structure (alias *Teg130*) with the presence of a C-rich exposed motif (364). As these motifs are a landmark of a category of sRNA in Firmicutes (177,178,365), RsaX20 was considered as a putative regulatory RNA (Fig. 37A).

**A**



**B**

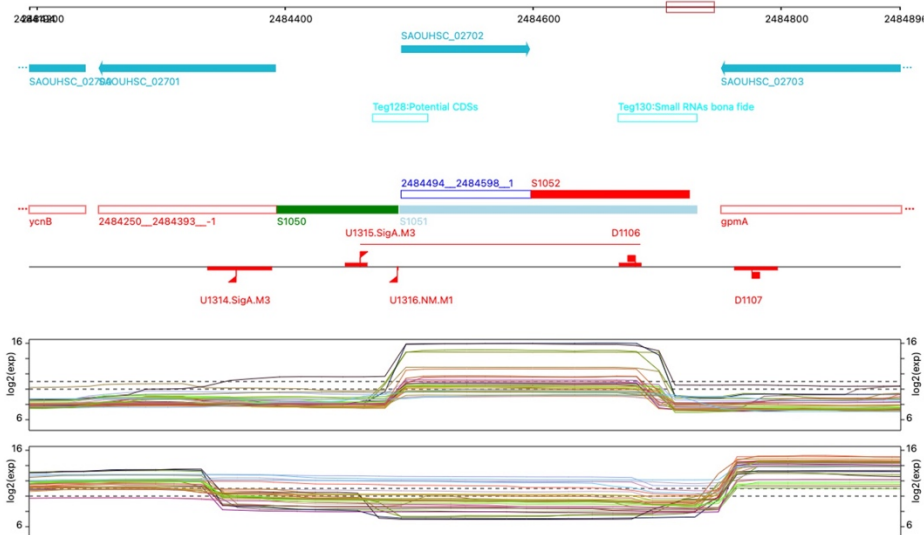


Figure 41. Structure and genomic location of RsaX20

A. Predicted secondary structure of RsaX20 (*Teg130*). C-rich exposed motif highlighted in red. Taken from (364). B. Genomic location and expression profile of RsaX20 (namely *Teg130* (364) or *S1052* (370)). Upper panel shows GenBank annotation (light blue, full), and annotation of transcription segments outside GenBank annotation (light blue, empty); Middle panel shows proposed annotation of transcription segments (full colored rectangles), and transcription units (red lines) with promoters as triangular flags and terminators as rectangular flags; Lower panel shows expression profile of WT *S. aureus* HG001 in 30 representative growth conditions. Taken from the *S. aureus* Expression Data Browser (427) (<http://genoscapist.migale.inrae.fr/aeb>).

Teg130 was predicted to be preceded by Teg128, a proposed sRNA located within the putative CDS SAOUHSC\_02702 (Fig. 37B). However, data from Mäder *et al* (HG001) and from our own transcriptomic study (NCTC8325) indicate that RsaX20 is in fact a single transcript of  $\approx 261$  nucleotides including both Teg128 and Teg130, possessing its own promoter and terminator sequence (Fig. 38 and 40).

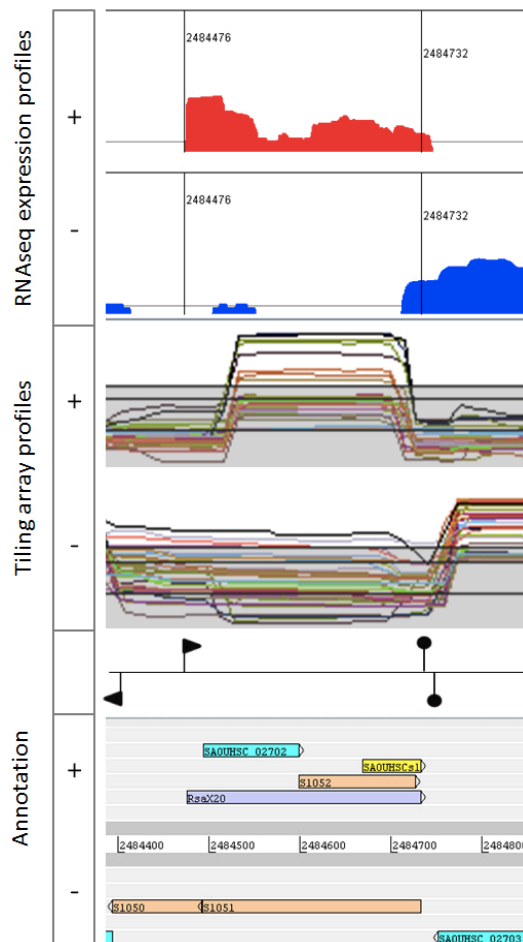


Figure 42. New proposed annotation for RsaX20.

Upper panel: Artemis viewer window showing read log-coverages from pooled RNA samples extracted from HG003 grown in 16 growth conditions. Middle panel: screen snapshots of tiling array data from HG001 grown in different conditions (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>, (370)). Lower panel: annotations including genomic coordinates and sRNA names from Carroll *et al.* (371) (yellow), Mäder *et al.* (370) (light orange) and Liu *et al.* (416) (mauve). Promoters (flags) and transcription terminators (hairpin loops) are placed according to Mäder *et al.* and/or TranstermHP software terminator predictions (428). Taken from (416).

Mäder *et al* also reported the presence of Zur binding motif upstream *rsaX20*. Zur, a Fur-like protein, is a zinc-dependent transcriptional regulator, responsible for regulation of several genes involved in zinc homeostasis in bacteria, including *S. aureus* (429,430). The presence of a Zur-

binding motif in the promoter region of *RsaX20* lead us to speculate the expression *RsaX20* would be indeed regulated *Zur* and moreover this putative sRNA could have a role in metal homeostasis in *S. aureus*.

## 2.1. *rsaX20* transcriptional regulation by *Zur*

As *rsaX20* is preceded by a *Zur* binding site, we consider that its expression would be *Zur*-dependent. To test that, the expression of *rsaX20* RNA was compared by Northern Blot, between a WT strain and a  $\Delta zur$  mutant at different growth stages. In the tested conditions, *RsaX20* was only observed in the  $\Delta zur$  mutant showing that the *rsaX20* gene is indeed repressed by *Zur*. We also observed that the amount of *RsaX20* decreased in post-exponential and stationary phases suggesting a down-regulation of *rsaX20* expression or a higher instability of *RsaX20* in these conditions (Fig. 39).

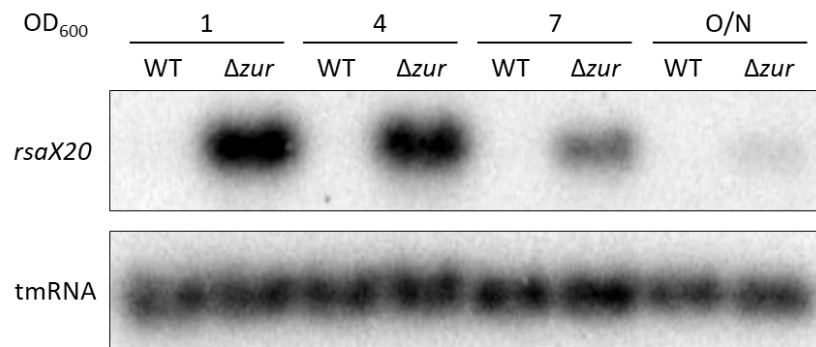


Figure 43. *rsaX20* regulation by *Zur*.

Northern blot showing levels of *rsaX20* with RNA samples obtained from a *S. aureus* WT strain and a  $\Delta zur$  mutant grown in rich media at OD<sub>600</sub> 1, 4, 7 and overnight. Expression of tmRNA is shown as a loading control.

This result confirms that *RsaX20* is part of the *Zur* regulon and in this sense, it might be involved in zinc homeostasis within *S. aureus*.

## 2.2. Does *rsaX20* encodes a protein?

A putative small ORF, sometimes annotated (*e.g.*, SAOUHSC\_02702 in NCTC8325), is indeed detected within *rsaX20*. In addition to a start and stop codons, SAOUHSC\_02702 possesses a canonical RBS, which could lead to the expression of a 35 amino-acid peptide (conserved in *S.*

*aureus* COL and USA300 strains under the name SACOL2414 and SAUSA300\_2361, respectively; according to Aureowiki database (431)) (Fig. 40). To test if this putative coding sequence was translated, we used a GFP reporter system. First, the complete sequence of RsaX20 with its promoter region was cloned into a multicopy plasmid. Then, the coding sequence of a GFP protein deprived of its start codon, was inserted in SAOUHSC\_02702, downstream and in frame with putative SAOUHSC\_02702 start codon ATG. The resulting plasmid was named p-02702::GFP. As a control of the experiment, a p-02702::GFP derivative with an altered SAOUHSC\_02702 start codon (ATG to TAG) was constructed by site directed mutagenesis. The resulting plasmid was named p-stop02702::GFP (Fig. 41A). The two constructed plasmids, p-stop02702::GFP and p-02702::GFP were transformed into a *S. aureus*  $\Delta$ zur strain and its isogenic parental strain.

TAAAACGTAATCATTACTATTTA CATATGAGTAGTTATCGGTCATAATATATTTACAAGGAGCGATAGAG  
ATGAAATTAGATTTACAGACTGCTCGTCGTAATTTAAATAGCCCTAACATTTAAAACAAGAAAGCGTGCTT  
TAAAGATTATTAAGCAACATAAAAGAGCGAAA TAA TTGTTTTAATTTGACTATATTGTATGTCTAACTAT  
ATTTTCGAGTATCCCTAGCTCGTTAATATAAACTCTATACTTAACTATTTTCCTTACATTACTTCACATAT  
TTAAGAGCTGCATATACACTCACTCCTTG

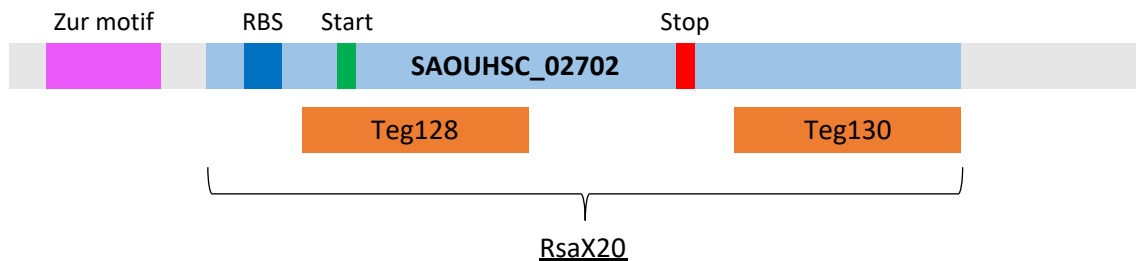


Figure 44. *rsaX20* sequence and organization.

Upper panel: *rsaX20* nucleotide sequence (underlined) with main features highlighted. SAOUHSC\_02702 ORF (bold); ribosome-binding site (blue); start codon (green); stop codon (red). Lower panel: graphic representation of *rsaX20* main features. *rsaX20* nucleotide sequence from *S. aureus* NCT8325 was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/> (432)) and Zur consensus sequence was obtained from Liu *et al* (416) as reported in RegPrecise (<https://regprecise.lbl.gov/> (417)).

No fluorescence was detected in strains proficient for Zur repression. However, the  $\Delta$ zur mutant with p-02702::GFP produced high intensity fluorescence, while the one with p-stop02702::GFP did not produce any (Fig. 41B).

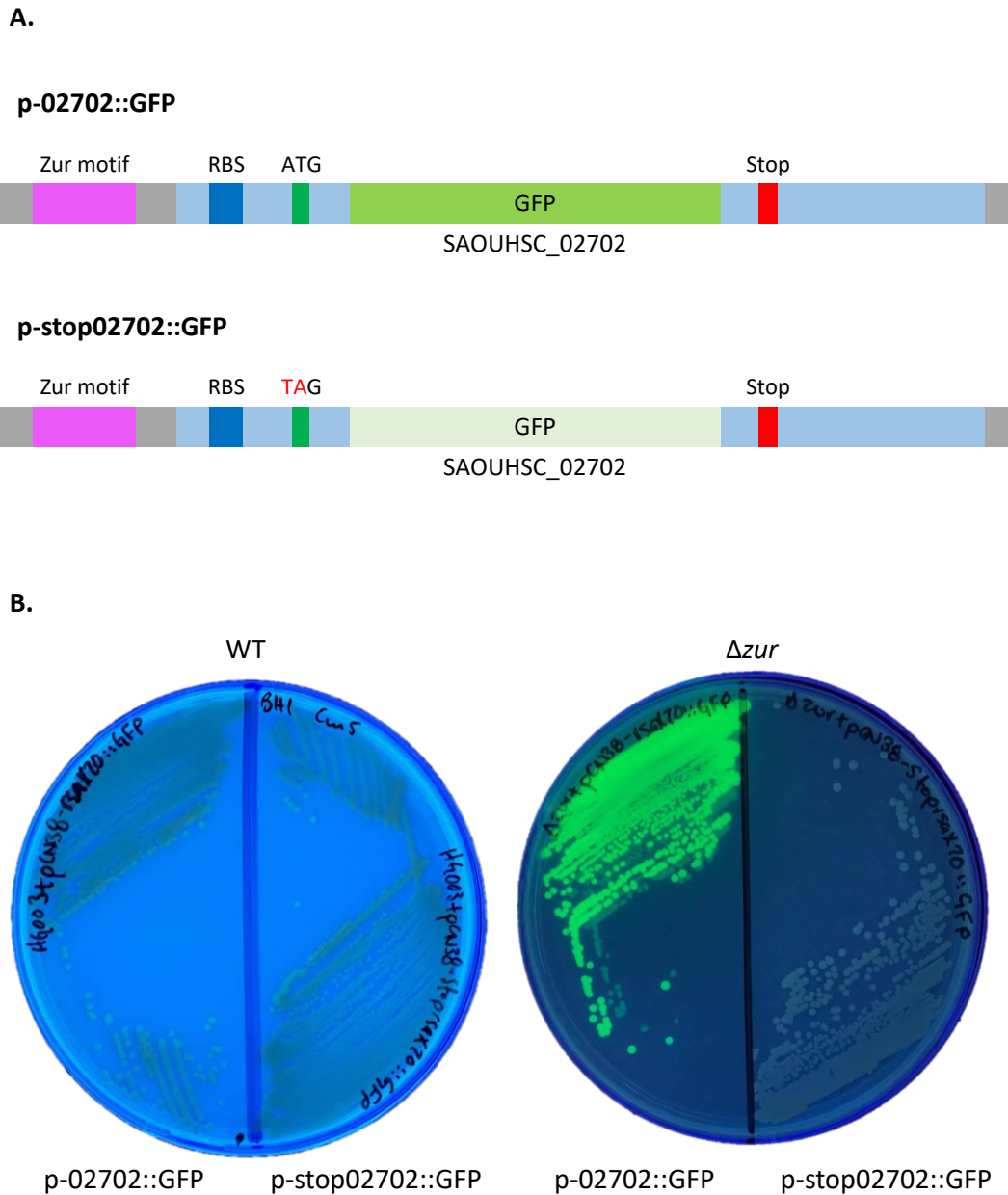


Figure 45. Zur-dependent expression of RsaX20.

A. Schematic representation of plasmids p-02702::GFP and p-stop02702::GFP. For p-02702::GFP, a *gfp* CDS deprived of its start codon was cloned in frame within 02702 which is under control of its endogenous promoter containing a Zur binding motif. Mutation of 02702 start codon (ATG>TAG) generated p-stop02702::GFP. B. Both plasmids were transformed either into a *S. aureus* WT strain or a  $\Delta zur$  mutant. High fluorescence was only observed with p-02702::GFP in the  $\Delta zur$  mutant, demonstrating that SAOUHSC\_02702 is likely a Zur-regulated CDS.

These experiments show that i) Zur represses the transcription of *rsaX20* and ii) SAOUHSC\_02702 is likely a coding sequence that produces a small peptide.



We conclude that RsaX20 is probably not a *bona fide* sRNA. When considering the amino acid composition of SAOUHSC\_02702, it is predicted to be a secreted polar peptide (<https://predictprotein.org/>), and importantly, it remains as a small protein with a potential role in metal homeostasis, particularly, within zinc homeostasis in *S. aureus*.

### 2.3. Methods

**Strains.** *S. aureus* NCTC 8325  $\Delta$ *zur* mutant strain was provided by Elise Borezee-Durant.

**Northern blots.** For culture growth conditions, RNA extraction and Northern blotting, see pg. 77. Probes for *IsrR*, *rsaX20* mRNA and tmRNA detection presented in pg. 124.

**GFP reporter system.** The complete sequence of RsaX20 with its promoter region was cloned into the multicopy plasmid pCN38. Then, the *gfp* coding sequence deprived of its start codon, was inserted in SAOUHSC\_02702, downstream and in frame with putative SAOUHSC\_02702 start codon ATG. The derivative plasmid with an altered SAOUHSC\_02702 start codon (ATG to TAG) was constructed by site directed mutagenesis using Gibson assembly.

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