FEBS Letters 589 (2015) 730–737

FEBS Letters

journal homepage: www.FEBSLetters.org

Involvement of major facilitator superfamily proteins SfaA and SbnD in staphyloferrin secretion in *Staphylococcus aureus*

Mélissa Hannauer [a], Jessica R. Sheldon [a], David E. Heinrichs [a,b,*]

[a] Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada
[b] Centre for Human Immunology, University of Western Ontario, London, Ontario N6A 5C1 Canada

ARTICLE INFO

Article history:
Received 5 December 2014
Revised 31 January 2015
Accepted 2 February 2015
Available online 11 February 2015

Edited by Renee Tsolis

Keywords:
Iron acquisition
Siderophores
Efflux

ABSTRACT

A paucity of information exists concerning the mechanism(s) by which bacteria secrete siderophores into the extracellular compartment. We investigated the role of SfaA and SbnD, two major facilitator superfamily (MFS)-type efflux proteins, in the secretion of the *Staphylococcus aureus* siderophores staphyloferrin A (SA) and staphyloferrin B (SB), respectively. Deletion of *sfaA* resulted in a drastic reduction of SA secreted into the supernatant with a corresponding accumulation of SA in the cytoplasm and a significant growth defect in cells devoid of SB synthesis. In contrast, *sbnD* mutants showed transiently lowered levels of secreted SB, suggesting the involvement of additional efflux mechanisms.

1. Introduction

Iron is an essential element participating in many biological processes like DNA, RNA and amino acid synthesis, and cellular respiration. Despite its high abundance on Earth, iron possesses low bioavailability at physiological pH. In order to access iron, many microorganisms produce and secrete low-molecular-weight, high-affinity iron-scavenging molecules called siderophores. While the biosynthesis and uptake mechanisms of siderophores have been widely studied, siderophore secretion mechanisms are not well characterized. At present, only two siderophore export systems have been identified in Gram-positive bacteria, YmfE and ExiT, and four in Gram-negative bacteria, EntS-AcrBD-MdtABC-TolC, PvdRT-OpmQ, CsbX and AlcS [1–7]. In Gram-negative bacteria, siderophores are first secreted into the periplasmic space, with subsequent export through the outer membrane by multi-domain efflux pumps. In the case of enterobactin, protochelin and alcaligin, siderophores secreted by *Escherichia coli*, *Azotobacter vinlandii* and *Bordetella* spp., respectively, MFS (Major Facilitator Superfamily)-type exporters are involved in siderophore translocation from the cytoplasm to the periplasm [3,6,8]. Enterobactin is subsequently secreted into the extracellular media by three RND (Resistance-Nodulation-Cell Division) type efflux pumps (AcrB, AcrD and MdtABC) associated with the outer membrane protein, TolC [4,5]. PvdRT-OpmQ, on the other hand, is an ATP-type efflux pump involved in pyoverdine secretion from the periplasm to the extracellular media in *Pseudomonas aeruginosa* [7]. In Gram-positive bacteria, active transport is required for passage of the siderophore through the cytoplasmic membrane. YmfE is an MFS-type efflux protein consisting of six transmembrane domains and is involved in bacillibactin secretion. Notably, a *ymfE* mutant displays only a 10% decrease in bacillibactin secretion, suggesting the involvement of other excretion systems for this siderophore [1]. Similar to PvdRT-OpmQ, ExiT is a member of the ABC-type transporters and is responsible for exochelin secretion in *Mycobacterium* spp. The mutation of this gene completely abolishes the production of exochelin, implying a coupling of the biosynthesis and export of this siderophore [2]. The majority of the efflux pumps characterized, to date, are encoded within the biosynthetic locus of their cognate siderophore.

Staphylococcus aureus produces two polycarboxylate-type siderophores staphyloferrin A (SA) and staphyloferrin B (SB) [9,10]. The genes involved in the biosynthesis of these two siderophores have been identified and are organized in two distinct

Abbreviations: SA, staphyloferrin A; SB, staphyloferrin B; MFS, major facilitator superfamily

* Corresponding author at: Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada. Fax: +1 (519) 661 3499.

E-mail address: deh@uwo.ca (D.E. Heinrichs).

http://dx.doi.org/10.1016/j.febslet.2015.02.002

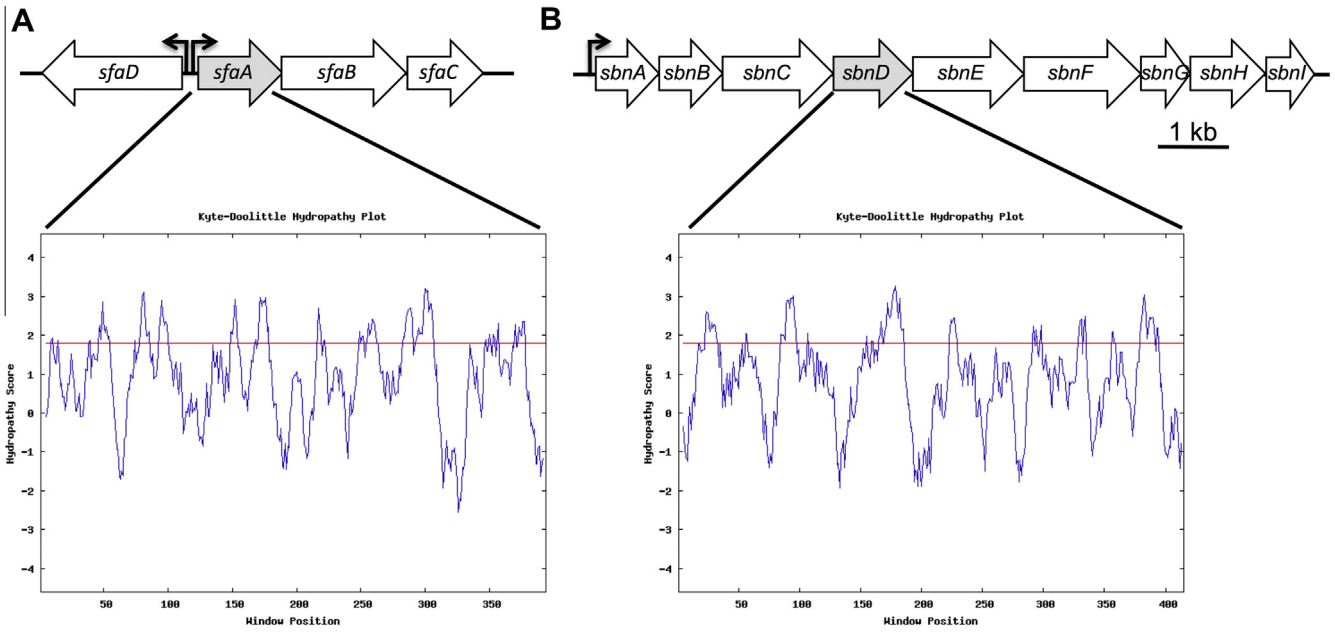

Fig. 1. Genetic context for *sfaA* and *sbnD*. The siderophore biosynthetic loci for SA (A) and SB (B) are shown, highlighting the genes encoding their corresponding efflux pumps. Displayed below the putative efflux pumps are hydropathy plots showing the prediction of 12 and 10 putative transmembrane domains (peaks above the red line) for SfaA and SbnD, respectively. The hydropathy plots were determined using the online prediction tool at http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm based on the Kyte-Doolittle method [29], with a window set to nine.

genetic loci (*sfa* and *sbn*) [11–14]. SA and SB biosynthesis occurs in the cytoplasm of the cell and is mediated by non-ribosomal peptide synthetase (NRPS)-Independent Synthesis (NIS) synthetases. Both siderophores are then secreted to the extracellular compartment were they bind iron from the environment or strip it from the host glycoprotein, transferrin. The iron-loaded siderophore is then transported back into the bacterium by two specific transporters, HtsABC (specific to SA), and SirABC (specific to SB) [15,16]. While the biosynthesis and uptake processes of the staphyloferrins have been extensively characterized [13,17], the mechanism by which both siderophores are secreted into the extracellular space remains unknown. The *sfa* and *sbn* operons both contain a gene coding for a putative efflux pump, *sfaA* and *sbnD*, respectively (Fig. 1). The presence of such a gene in each of the siderophore biosynthetic loci strongly suggested their involvement in the secretion of the associated siderophore, but their actual involvement in SA and SB secretion has never been demonstrated. In this study, we examined the role of SfaA and SbnD, two MFS-type efflux proteins, in SA and SB secretion.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

Bacterial strains and plasmids used in this study are summarized in Table 1. *E. coli* strains were cultured in Luria–Bertani broth (LB; BD Canada, Mississauga, ON, Canada), and *S. aureus* strains were cultured in tryptic soy broth (TSB; BD Canada). Iron-limited growth was performed in Tris Minimal Succinate (TMS) medium [18], treated with 5% (wt/vol) Chelex-100 resin (Bio-Rad, Mississauga, ON, Canada) for 24 h (C-TMS). All media were prepared using Milli-Q purified water. For plasmid selection, the following antibiotics were added: ampicillin (100 μg/mL) for *E. coli*, chloramphenicol (10 μg/mL) for *S. aureus*.

2.2. Mutant construction and complementation

Single gene deletions of each *sfaA*, *sbnD*, and *norA* were performed using the pKOR1 plasmid, as described previously [19], and the primers listed in Table 2. Gene deletions were confirmed by sequencing PCR amplicons from the chromosome of mutants. For complementation of the *sfaA* mutant phenotype, the *sfaA* gene including its native promoter was amplified with primers listed in Table 2 and cloned into pLI50 [20]. For complementation of the *sbnD* mutant, the *sbnD* gene was amplified and cloned into the pALC2073 vector [21].

2.3. Bacterial growth curves

Cells were pre-cultured overnight in C-TMS. Cells were then washed and inoculated to an OD_{600} equivalent of 0.005 in 20 mL of C-TMS containing 20% horse serum. Cultures were incubated in 250-mL erlenmeyer flasks (previously treated with 0.1 M HCl) over 24–36 h, with shaking at 220 rpm at 37 °C. Samples were removed for OD_{600} measurements every 2 h. For inducing expression of *sbnD* from pALC2073, 100 ng/mL of anhydrotetracycline was added to the media.

2.4. Plate bioassays

Siderophore production by *S. aureus* RN6390 and its isogenic mutants was monitored using disk diffusion plate bioassays as previously described [22]. Concentrated supernatants were prepared as described [23]. To extract the cytoplasmic fraction, the corresponding cell pellet was washed once with saline and then resuspended in 500 μL of digestion buffer (50 mM Tris HCl; 145 mM NaCl; 1 mg/mL iodoacetamide; 50 μg lysostaphin). After overnight incubation at 37 °C, the volume was adjusted to 2.5 mL with MilliQ water prior to lysis with a TS HAIVA cell disruptor (Constant

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Genotype/phenotype[a]	Source or reference
S. aureus		
RN6390	Prophage-cured wild-type strain	[31]
RN4220	$r_K^- m_K^+$; accepts foreign DNA	[30]
H306	RN6390 Δ*sirA*::Km; Km^R	[12]
H1324	RN6390 Δ*sbn*::Tc; Tc^R	[13]
H1448	RN6390 Δ*htsA*::Tc; Tc^R	[13]
H1661	RN6390 Δ*sfa*::Km; Km^R	[13]
H1649	RN6390 Δ*sfa*::Km Δ*sbn*::Tc; Km^R Tc^R	[13]
H2550	RN6390 Δ*sbnD*	This study
H2562	RN6390 Δ*sbnD* Δ*sfa*	This study
H2612	RN6390 Δ*norA*	This study
H2719	RN6390 Δ*sfaA*	This study
H2722	RN6390 Δ*sfaA* Δ*sbn*::Tc; Tc^R	This study
H2733	RN6390 Δ*sbnD* Δ*sfa* Δ*norA*	This study
E. coli		
DH5α	φ_{80} d*LacZ*Δ*M15 recA1 endA1 gyrA96 thi-1 hsdR17*($r_K^- m_K^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*) *U169*	Promega
Plasmids		
pKOR1	Temperature sensitive *E. coli*/*S. aureus* suicide shuttle vector; Ap^R Cm^R	[19]
pKOR1Δ*sfaA* (pMH38)	pKOR1 containing DNA homologous to the 5′- and 3′-flanking regions of *sfaA* for markerless deletion	This study
pKOR1Δ*sbnD* (pJS10)	pKOR1 containing DNA homologous to the 5′- and 3′-flanking regions of *sbnD* for markerless deletion	This study
pKOR1Δ*norA* (pMH24)	pKOR1 containing DNA homologous to the 5′- and 3′-flanking regions of *norA* for markerless deletion	This study
pLI50	*E. coli*/*S. aureus* shuttle vector; Ap^R Cm^R	[20]
psfaA	pLI50 derivative containing *sfaA* from *S. aureus*; Cm^R	This study
pALC2073	*E. coli*/*S. aureus* shuttle vector; tetracycline-inducible promoter; Ap^R Cm^R	[21]
psbnD	pALC2073 derivative containing *sbnD* from *S. aureus*; Cm^R	This study
pGYLux	*E. coli*/*S. aureus* luciferase reporter vector; Ap^R Cm^R	[24]
p*sfaA*Lux	pGYLux containing *sfaA-sfaD* promoter in the forward orientation	This study
p*sfaD*Lux	pGYLux containing *sfaA-sfaD* promoter in the reverse orientation	This study
p*sbnA*Lux	pGYLux containing *sbnA* promoter	This study

[a] *Abbreviations:* Ap^R, Cm^R, Km^R and Tc^R resistance to ampicillin, chloramphenicol, kanamycin and tetracycline, respectively.

Table 2
Oligonucleotides used in this study.

Purpose	Sequence[a]
Markerless deletion of *sfaA*	Upstream fragment: GGGGACAAGTTTGTACAAAAAAGCAGGCTAGATAAACCAGCATAATAATCCACC (Forward) TGTCATAAAACTTACACCCGC (Reverse) Downstream fragment: /5Phos/GATAGAGGTATGATTCCATTGGG (Forward) GGGGACCACTTTGTACAAGAAAGCTGGGTGAAAGTATTGTAAACGGCG (Reverse)
Markerless deletion of *sbnD*	Upstream fragment: GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTCGTCTTCAAGTATGCGTTC (Forward) TCACCATTGGATTTGGTACGG (Reverse) Downstream fragment:/5Phos/ACCATCACTAATCAAATCAACG (Forward) GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGGTTCAACTGTTTGCC (Reverse)
Markerless deletion of *norA*	Upstream fragment: GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCAATCCAAATGTACCTG (Forward) GTGAACGGATGTATAGATGATCG (Reverse) Downstream fragment: /5Phos/CCGTAACATTTATAGTCAGCATTG (Forward) GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAGGTCAGAAACAACGC (Reverse)
Cloning of *sfaA* for complementation	GATCGGTACCATGTGACCAATCTCTGAGTGAC (Forward, KpnI) GATCGAGCTCGAAATAAATGTTTGCTCGGG (Reverse, SacI)
Cloning of *sbnD* for complementation	GATCGGTACCCAAATCCAATGGTGACATATG (Forward, KpnI) ATCGAGCTCCGCTGCATGTTGTATTAATTC (Reverse, SacI)
Cloning of *sfaA-D* promoter region	GATCCCCGGGGATGTGACCAATCTCTGAG (Forward, SmaI) GATCCCCGGGGCCCTATTTGTCCAATCC (Reverse, SmaI)
Cloning of *sbn* promoter region	GATCCCCGGGTCAATAAAATATTTATGATTTACATGC (Forward, SmaI) GACTCCCGGGAACTTGCTTCCATAACTGCAATT (Reverse, SmaI))
rpoB RT-PCR	AGAGAAAGACGGCACTGAAAACAC (Forward) ATAACGACCCACGCTTGCTAAG (Reverse)
sfaC RT-PCR	ATGGTCAAGGACACTATC (Forward) AAGTAGCGTATCAATCTTTG (Reverse)
sfaD RT-PCR	CCTCTAATGCAATGCCATATTTA (Forward) ACAATGAATCACCTATCGTGACA (Reverse)
iruO RT-PCR	GGAGGAAATACAGCATTAGATTGG (Forward) TTCATCATCATTGCCGACC (Reverse)

[a] Restriction sites for cloning or *attB1*/*attB2* recombination sites are underlined.

Systems Ltd., UK). For the assay, 15 μL of concentrated supernatant or 30 μL of extracted cytoplasm were used [22]. Reporter strains RN6390Δ*htsA* or RN6390Δ*sirA* were used to detect the presence of SB and SA, respectively. Growth promotion was determined by measuring the radius of growth around the disk after 24 h incubation at 37 °C.

2.5. Luciferase assay

The promoter regions of *sfa* and *sbn* were amplified using the primers described in Table 2 and cloned into the pGYlux reporter vector [24]. The plasmids were then introduced into wild-type *S. aureus* and the Δ*sfaA* and Δ*sbnD* mutants. Stationary-phase cultures grown overnight in iron-restricted media were inoculated to a starting OD_{600} of 0.1 in iron-restricted media and grown until an OD_{600} of approximately 1.0. Luminescence was assessed by aliquoting 100 μL of each culture in a white 96-well plate and reading the emission at 490 nm, and OD_{600} was similarly determined at the same timepoints using a clear 96-well plate. Both plates were read in a Synergy H4 microtiter plate reader (BioTek, USA).

2.6. Real time PCR

Cells were prepared as described above for the luminescence assay. RNA was extracted following the Aurum™ total RNA Mini Kit instructions and as described in [23]. 500 ng of RNA was reverse transcribed using SuperScript® II reverse transcriptase from Invitrogen. PCR amplification was performed in a Rotor-Gene 6000 Real Time Analyzer (Corbett Life science) using the iQ™ SYBR® Green supermix from Bio-Rad and the primers listed Table 2. For quantification, samples were normalized to the housekeeping gene *rpoB*.

3. Results

3.1. sfaA and sbnD mutations affect growth in an iron-deficient growth medium

SfaA and SbnD are putative MFS-type efflux pumps, encoded from within the SA and SB biosynthetic operons, by *sfaA* and *sbnD*, respectively (Fig. 1). Sequence analyses place these two proteins within this family because they each contain the five conserved motifs (motifs A, B, C, D2 and G) representative of MFS efflux pumps [25]. Kyte–Doolittle plots (Fig. 1) and TMPred predict SfaA has 12 transmembrane domains, while SbnD has 10 transmembrane domains. Searches of the databases using HHPRED identified significant similarity of each of the proteins to MFS-type proteins involved in the import of di/tri-peptides, phosphate, glycerol-3-phosphate, and the *E. coli* EmrD pump, involved in resistance to energy uncouplers and detergents [26,27].

To investigate the role of the putative efflux pumps in the secretion of SA and/or SB, we generated in-frame deletions of each gene in the *S. aureus* chromosome. As shown in Fig. 2A, no discernable growth defect, in comparison to the wild-type strain RN6390, was observed for the Δ*sfaA* mutant (H2719) when grown in iron-restricted media. This would be in agreement with previous studies demonstrating that deletion of the SA biosynthetic locus does not hinder growth of *S. aureus* in iron-restricted media [13]. However, when we introduced the Δ*sfaA* mutation into a strain debilitated for SB biosynthesis, generating strain H2722 (i.e. Δ*sbn* Δ*sfaA*), the deletion of *sfaA* now resulted in a growth defect that was comparable to the siderophore biosynthetic mutant strain (i.e. Δ*sbn* Δ*sfa* background; H1649) (Fig. 2A). Growth of the Δ*sbn* Δ*sfaA* mutant was restored to that similar to the Δ*sbn* (H1324) strain when *sfaA* was expressed *in trans*.

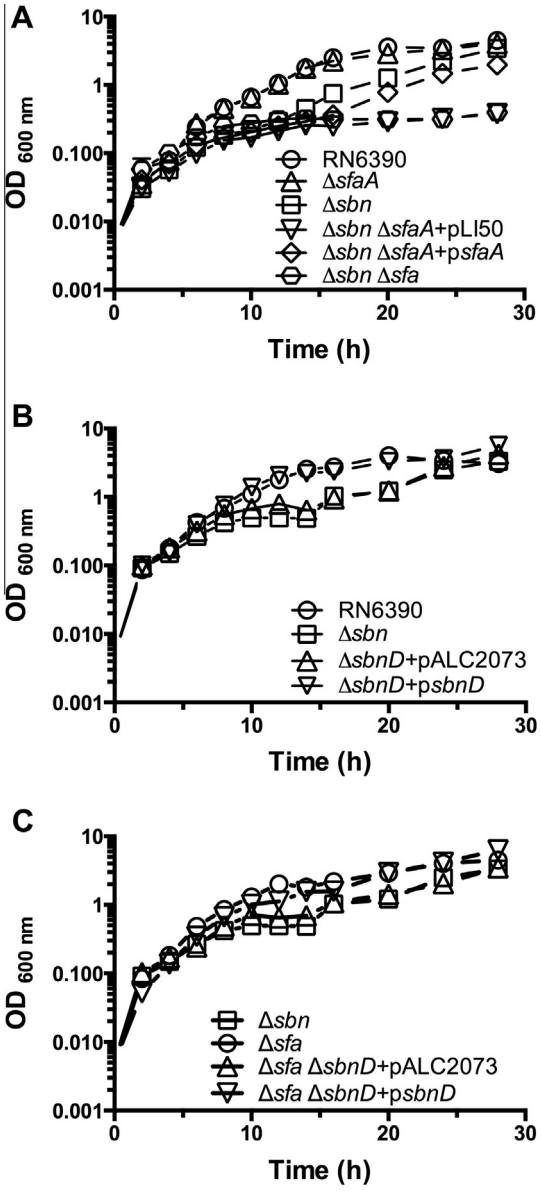

Fig. 2. Deletion of *sfaA* and *sbnD* impacts growth in iron-depleted media. For all panels, the *S. aureus* wild-type strain (RN6390) and/or its isogenic mutants were grown in C-TMS containing 20% horse serum. The error bars represent standard deviation of the mean; $n = 3$. Data are representative of 3 independent experiments.

Mutation of the gene encoding SbnD (strain H2550) resulted in a growth delay similar to a SB-deficient strain (Δ*sbn*; H1324) (Fig. 2B). The growth of this mutant is restored to wild-type levels when *sbnD* is expressed *in trans*. However, in contrast to the growth defect observed for the Δ*sbn* Δ*sfaA* mutant, no further defect was observed when the Δ*sbnD* mutation was inserted into an SA-deficient strain (Δ*sfa* Δ*sbnD*; H2562) (Fig. 2C).

3.2. SA secretion is defective in an sfaA mutant

Given that the Δ*sfaA* and Δ*sbnD* mutations had a negative effect on iron-starved growth, we next tested whether this growth defect was due to an alteration in the ability of the siderophores to be secreted into the growth medium. The presence of siderophores in either the intracellular compartment, or the extracellular medium, was assessed using defined reporter strains in a disk diffusion plate bioassay. As shown in Fig. 3A, after growth in

iron-restricted TMS medium, the concentrated, spent culture supernatants of wild-type RN6390, a Δ*sfaA* mutant (H2719), and the complemented Δ*sfaA* mutant all promote growth of the wild-type RN6390 reporter strain. In contrast, the spent culture supernatant of the Δ*sfaA* mutant, but not wild-type (RN6390) or the complemented mutant, promotes significantly less growth of the Δ*sirA* reporter strain (H306). This result indicates that the spent culture supernatant of the Δ*sfaA* mutant contains significantly lower levels of SA than the other two strains. The decrease in SA in the supernatant is correlated with a significant increase in the amount of SA accumulating in the cytoplasm of the Δ*sfaA* mutant, as the cytoplasmic extract of this mutant enhances the growth of the Δ*sirA* reporter strain, relative to the wild-type or complemented strains (Fig. 3B). No difference in SB levels was observed between the Δ*sfaA* mutant and wild-type (data not shown).

To date, the siderophore secretion systems described in Gram-positive bacteria involve either several efflux pumps, where deletion of one system results in only a partial defect in siderophore secretion [1], or a coupling of siderophore secretion and biosynthesis, where the disruption of siderophore secretion leads to negative feedback on the expression of the biosynthesis genes [2]. In order to determine whether cytoplasmic SA accumulation in the Δ*sfaA* mutant had an impact on gene expression, we cloned the promoter region located between *sfaA* and *sfaD* (Fig. 1) in both orientations in a luciferase reporter vector. The luciferase activity of the two resulting reporter vectors was monitored in the wild-type and Δ*sfaA* mutant strains during exponential phase growth in iron-restricted media. The results show no difference in the luciferase activity in the Δ*sfaA* mutant when compared to wild-type strain (Fig. 3C), indicating that the promoter activities for the SA biosynthetic genes are not affected by the intracellular accumulation of SA. Since promoter activity is not necessarily reflective of transcripts levels, we performed qRT-PCR to determine the levels of transcripts in presence of accumulated SA. As depicted in Fig. 3D, transcript levels in the Δ*sfaA* mutant were significantly decreased for both *sfaC* and *sfaD* genes. To confirm that SA accumulation wasn't having a global effect on RNA levels in the cell, we demonstrated that transcript levels for *iruO*, an unrelated iron-regulated gene, were unaffected in the Δ*sfaA* mutant compared to WT. The decreased transcript levels in the Δ*sfaA* strain, however, are clearly sufficient to allow SA synthesis as demonstrated above.

3.3. SB secretion is partially affected in an sbnD-deficient strain

The distinctive Δ*sbn*-like growth phenotype observed for the Δ*sbnD* mutant (H2550) led us to hypothesize that the secretion of SB might be affected during the early stages of growth. To determine if SB secretion is delayed in the Δ*sbnD* mutant, we harvested bacterial culture supernatants at early and late growth phases, and

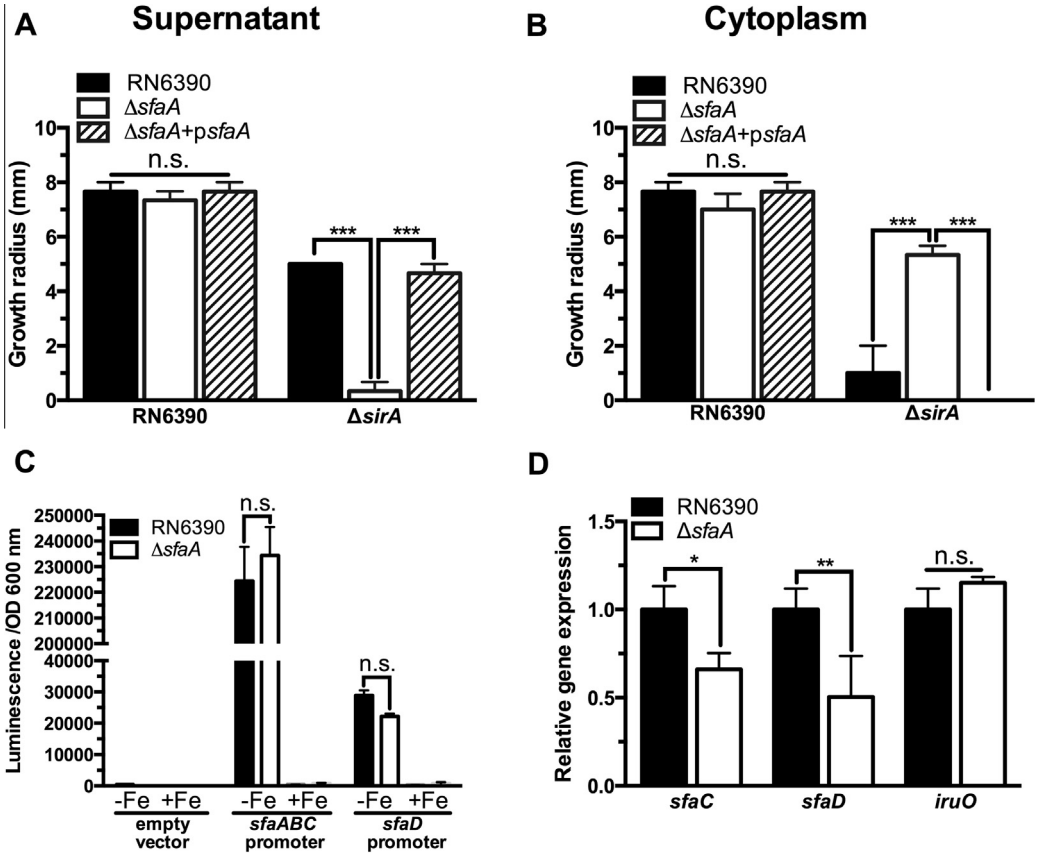

Fig. 3. *sfaA* mutants accumulate SA in the cytoplasm. Plate disk diffusion growth assays were performed using the concentrated supernatants (A) or cytoplasmic extracts (B) of strains, as indicated in the legends. Plates were seeded with WT or a Δ*sirA* reporter strains (indicated on the *x*-axes). Growth promotion is measured as the radius of growth (mm) around each disk impregnated with samples of either concentrated supernatants or cytoplasmic extracts. (C) Luciferase assays were performed with the *sfaA-D* promoter fragment cloned upstream of promoterless luciferase genes. The fragment was inserted into the plasmid in both orientations in order to assess *sfaABC* and *sfaD* promoter activities. (D) quantitative RT-PCR was performed on *sfaC*, the divergently encoded *sfaD* gene and the unrelated *iruO* gene, using primers listed in Table 2. Gene expression in Δ*sfaA* was compared to expression in wild-type, which was arbitrarily set to 1. Data were normalized to the housekeeping gene *rpoB* and are representative of qPCR performed on at least three independent RNA extractions. Error bars represent standard deviation of the mean; $n = 3$. Data are representative of 3 independent experiments. Statistical significance was determined by Student's *t*-test; ns, not significant, $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$.

concurrently extracted the cytoplasm of the same cells. As shown in Fig. 4A, after 14 h of growth, the supernatant harvested from the Δ*sbnD* mutant shows significantly lower bioassay positive activity than the wild-type supernatant. This decrease in SB secretion is correlated with an increased intracellular accumulation of SB (Fig. 4A, right panel). This defect in SB secretion at an early growth stage likely explains the growth delay observed in Fig. 2A. After 36 h of growth, no difference in SB secretion or intracellular SB accumulation is observed between the wild-type strain and the Δ*sbnD* mutant (Fig. 4B). These results suggest that the Δ*sbnD* mutant is still capable of secreting SB. Finally, as with the lack of an effect of Δ*sfaA* on promoter activity for the biosynthetic genes, we found that *sbnA* promoter activity was not altered in response to mutation of *sbnD* during iron-restricted growth (Fig. 4C).

3.4. Mutation of NorA does not affect SB-dependent growth

A potential candidate for SB secretion is the MFS-type efflux pump NorA, since a recent study showed that NorA expression was influenced by iron levels in the growth medium [28]. Given that we observed no major growth defect due to the Δ*sbnD* mutation in the SA-deficient strain (Fig. 2C), we combined the Δ*sbnD* Δ*sfa* mutations with a Δ*norA* mutation, generating strain H2733, and monitored the growth of this mutant in iron-restricted media. As shown in Fig. 5, the Δ*norA* mutant (H2612) displayed growth kinetics similar to the wild-type strain, and the combination of the Δ*norA* mutation with the Δ*sbnD* Δ*sfa* mutations (H2733) did not demonstrate an exacerbated growth defect relative to that of the Δ*sbnD* Δ*sfa* strain (H2562). Consistent with the growth data (Fig. 5A), the Δ*norA* mutant was not affected for the secretion of

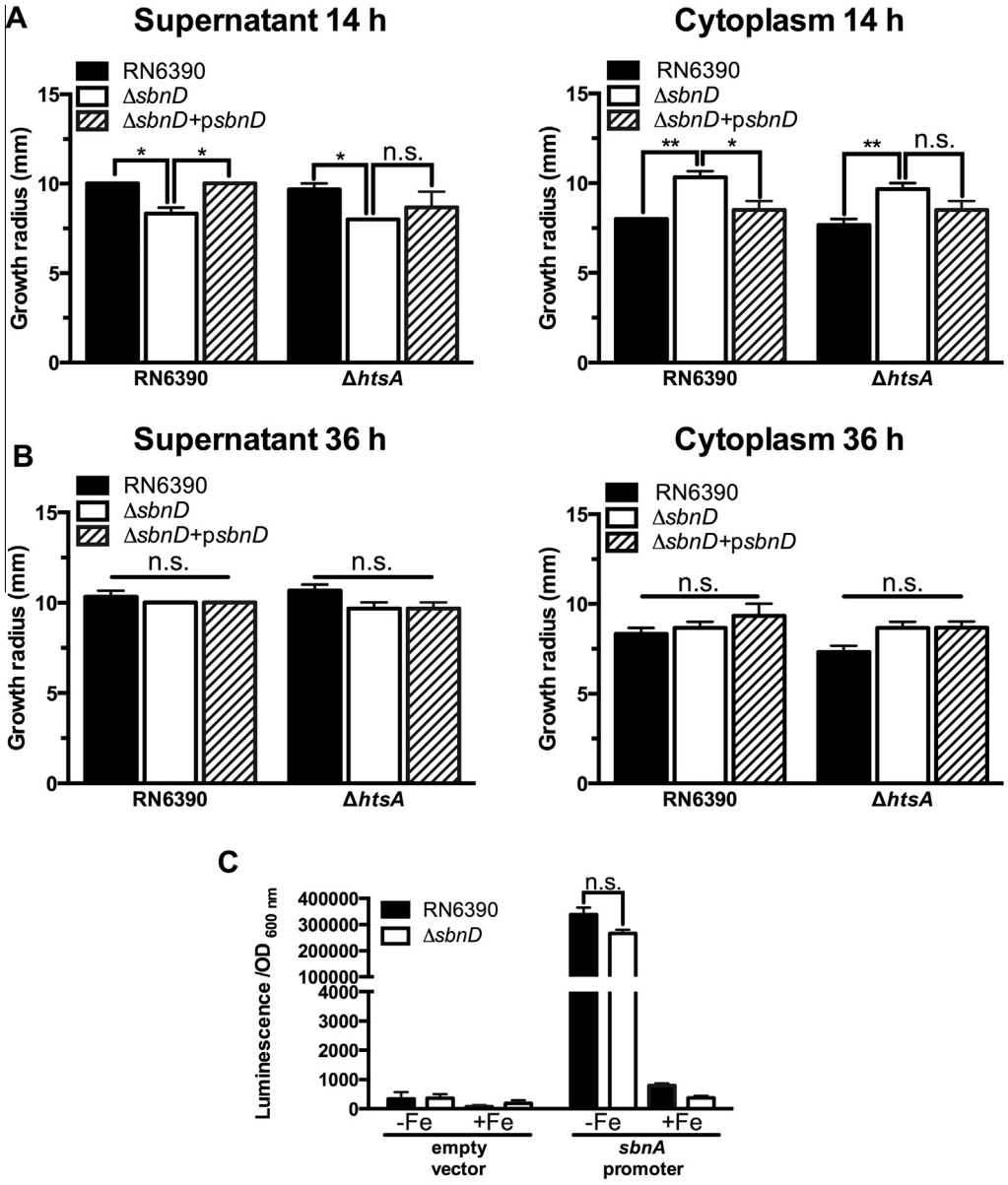

Fig. 4. *sbnD* mutants are partially impaired for SB secretion. Plate disk diffusion growth assays were performed using the concentrated supernatants (A) or cytoplasmic extracts (B) of the indicated strains. Plates were seeded with WT or Δ*htsA* reporter strains, as indicated on the *x*-axis. Growth promotion was measured as the radius of growth (mm) around each disk impregnated with either concentrated supernatant or cytoplasmic extract. (C) luciferase assays were performed with the *sbnA* promoter cloned upstream of promoterless luciferase genes. Error bars represent standard deviation of the mean; $n = 3$. Data are representative of 3 independent experiments. Statistical significance was determined using Student's *t*-test; ns, not significant, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$.

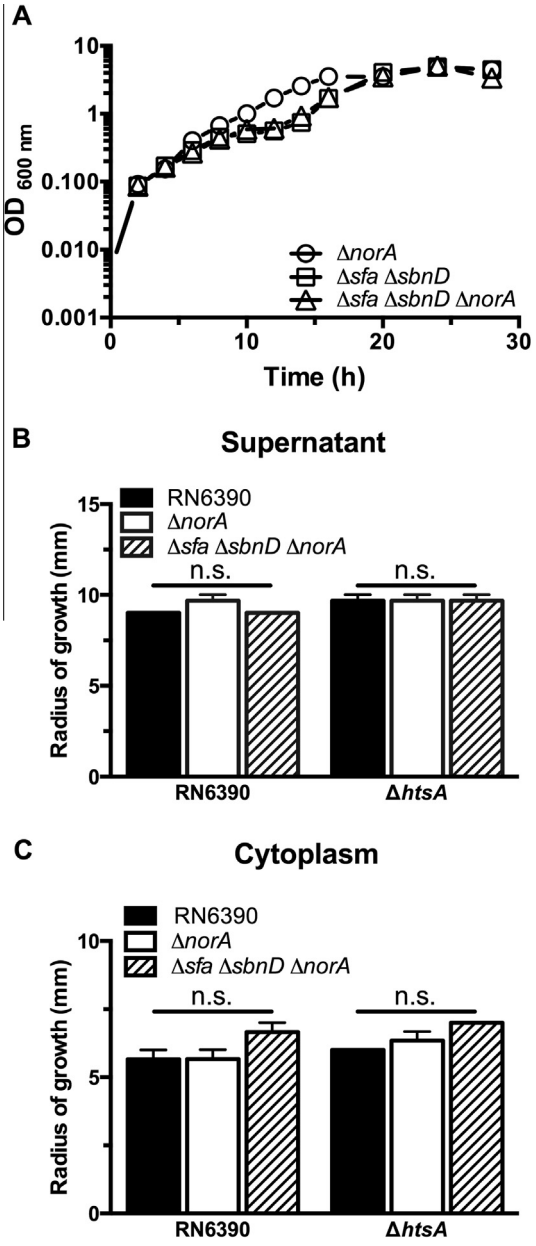

Fig. 5. NorA is not involved in SB-dependent, iron-restricted growth of *S. aureus*. (A) *S. aureus* strains, as indicated, were grown in C-TMS containing 20% horse serum. In panels B and C, disk diffusion bioassays were performed using the concentrated supernatants (B) or cytoplasmic extracts (C) of the indicated strains. Plates were seeded with WT or Δ*htsA* reporter strains, as indicated on the *x*-axis. Growth promotion was measured as the radius of growth (mm) around each disk impregnated with either concentrated supernatant or cytoplasmic extract. Data are representative of 3 independent experiments.

SB based on bioassays performed on spent culture supernatants and extracted cytoplasms (Fig. 5B and C), supporting the suggestion that NorA is not involved in SB secretion.

4. Discussion

The siderophore secretion mechanisms of both Gram-negative and Gram-positive bacteria have remained largely uncharacterized, including for SA and SB, the two staphyloferrin siderophores produced by *S. aureus*. In this study, we investigated the role of SfaA and SbnD, encoded from within the SA and SB biosynthetic loci, respectively (Fig. 1) and a third genetically-unlinked MFS-type efflux protein, NorA, in the secretion of the staphyloferrins.

The most significant finding from this study was our demonstration that SfaA appears solely responsible for SA secretion and that in its absence SA accumulates in the *S. aureus* cytoplasm. A previous study on AlcS, the alcaligin secretion protein in *Bordetella* spp., also describes a decrease in the amount of siderophore secreted to the extracellular milieu, correlated with increased cell-associated siderophore [6]. The authors also discuss a regulatory link between siderophore biosynthesis and secretion [6]. Similarly, disruption of the secretion mechanisms for exochelin in *Mycobacterium smegmatis* and pyoverdine in *P. aeruginosa* resulted in either a complete inhibition of siderophore biosynthesis, as described for exochelin [2], or a decrease in the biosynthesis, as shown for pyoverdine [7]. In the latter case, the authors suggested that the accumulation of pyoverdine or pyoverdine precursors in the periplasm of the cell could block the active site of the biosynthetic enzymes thus causing a premature arrest in biosynthesis [7]. Furthermore, cytoplasmic accumulation of siderophores could also drastically alter the iron content of the cell through the sequestration of intracellular iron, thus hindering the function of iron-sulfur cluster containing enzymes. Accordingly, downregulation of siderophore synthesis would be beneficial to avoid potentially toxic intracellular accumulation in the absence of secretion. However, our data demonstrate that while SA accumulates in an Δ*sfaA* mutant, no effect on the ability of the strain to grow in iron-restricted media is noticed. First, the data demonstrate that the strain is still using SB to support iron-restricted growth, since an Δ*sfaA* Δ*sbn* mutant grows extremely poorly in the same media. Second, and possibly more importantly, the data demonstrate that the intracellular accumulation of SA does not adversely affect the fitness of *S. aureus*. We further tested this notion by examining sensitivity of the Δ*sfaA* mutant to oxidative stress. We found no increased sensitivity, relative to WT, to exposure to hydrogen peroxide (data not shown). Nevertheless, while cytoplasmic SA accumulation had no effect on the promoter activity of *sfa* transcripts, a reduction of the transcript levels was observed, probably elicited by decreased RNA stability. Although we have not yet determined the mechanism underpinning this observation, it would be reasonable to hypothesize that one mechanism by which to lower stress induced by intracellular SA accumulation is through negative feedback on SA synthesis, possibly through mechanisms related to RNA stability.

Unlike SA secretion, the mechanism of SB secretion seems more complex and likely involves more than one efflux protein. Indeed, although the loss of SbnD resulted in a growth delay identical to that of a SB biosynthetic mutant (Fig. 2B and C), only a slight reduction in SB secretion was observed, and only at an early growth stage (Fig. 3A). This phenotype is similar to what has previously been observed for bacillibactin secretion, where only a slight defect in siderophore secretion was described, implying that more than one efflux pump is required for export of this siderophore. Moreover, the recent identification of three RND-type efflux pumps as being involved in the secretion of enterobactin in *E. coli* (AcrAB/AcrAD and MtdABC) solidifies the hypothesis that several export systems can be involved in the secretion of a single siderophore [5].

The possibility remains that the intracellular accumulation of SB in an Δ*sbnD* mutant may be toxic, and that the underwhelming phenotype for SB secretion may be attributable to the release of SB into the extracellular media upon cell death, which would promote the growth of sister cells in the medium. However, we think this is unlikely given that SA accumulation did not appear to significantly impact intracellular iron metabolism in Δ*sfaA* mutants. We favor the hypothesis that, in addition to SbnD, there exist alternate routes of SB secretion out of the cell. Unfortunately, we have not yet been able to identify which combination of efflux pumps are involved in SB secretion. Combining Δ*sbnD* with either Δ*norA*, Δ*sfaA*, or Δ*norA* and Δ*sfaA* in the same cell did not significantly

alter SB secretion or SB-dependent growth of *S. aureus* over and above the effect contributed by the Δ*sbnD* mutation alone. If NorA is involved in SB secretion, then its activity is still masked by the activity of alternate secretion proteins. Recent findings have revealed that SA and SB are differentially synthesized in response to available carbon sources, where SA production is hindered in the presence of glucose due to the decreased availability of siderophore precursor molecules [23]. As such, it is believed that SB is the predominant siderophore produced by *S. aureus* during systemic infections, where serum glucose would limit SA production. In this context, it is not unreasonable to assume that more than one efflux pathway would be available to ensure the secretion of SB in the iron-restricted host.

This is the first study to investigate the secretion pathways of two endogenous siderophores produced by the same organism. Here we were able to determine that SfaA is likely the sole efflux protein involved in SA secretion, whereas secretion of SB involves SbnD and as yet unidentified transporters. The genome of *S. aureus* encodes numerous putative efflux proteins, and we are currently investigating the role of other iron-regulated efflux proteins in the secretion of SB.

Acknowledgements

The authors would like to thank Cristina Marolda for providing the *sbn* promoter-lux fusion plasmid. This work was supported by an operating grant to DEH from the Canadian Institutes of Health Research (CIHR).

References

[1] Miethke, M., Schmidt, S. and Marahiel, M.A. (2008) The major facilitator superfamily-type transporter YmfE and the multidrug-efflux activator Mta mediate bacillibactin secretion in *Bacillus subtilis*. J. Bacteriol. 190, 5143–5152.
[2] Zhu, W., Arceneaux, J.E., Beggs, M.L., Byers, B.R., Eisenach, K.D. and Lundrigan, M.D. (1998) Exochelin genes in *Mycobacterium smegmatis*: identification of an ABC transporter and two non-ribosomal peptide synthetase genes. Mol. Microbiol. 29, 629–639.
[3] Furrer, J.L., Sanders, D.N., Hook-Barnard, I.G. and McIntosh, M.A. (2002) Export of the siderophore enterobactin in *Escherichia coli*: involvement of a 43 kDa membrane exporter. Mol. Microbiol. 44, 1225–1234.
[4] Bleuel, C., Große, C., Taudte, N., Scherer, J., Wesenberg, D., Krauß, G.J., Nies, D.H. and Grass, G. (2005) TolC is involved in enterobactin efflux across the outer membrane of *Escherichia coli*. J. Bacteriol. 187, 6701–6707.
[5] Horiyama, T. and Nishino, K. (2014) AcrB, AcrD, and MdtABC multidrug efflux systems are involved in enterobactin export in *Escherichia coli*. PLoS One 9, e108642.
[6] Brickman, T.J. and Armstrong, S.K. (2005) *Bordetella* AlcS transporter functions in alcaligin siderophore export and is central to inducer sensing in positive regulation of alcaligin system gene expression. J. Bacteriol. 187, 3650–3661.
[7] Hannauer, M., Yeterian, E., Martin, L.W., Lamont, I.L. and Schalk, I.J. (2010) An efflux pump is involved in secretion of newly synthesized siderophore by *Pseudomonas aeruginosa*. FEBS Lett. 584, 4751–4755.
[8] Page, W.J., Kwon, E., Cornish, A.S. and Tindale, A.E. (2003) The *csbX* gene of *Azotobacter vinelandii* encodes an MFS efflux pump required for catecholate siderophore export. FEMS Microbiol. Lett. 228, 211–216.
[9] Konetschny-Rapp, S., Huschka, H.G., Winkelmann, G. and Jung, G. (1988) High-performance liquid chromatography of siderophores from fungi. Biol. Met. 1, 9–17.
[10] Drechsel, H., Freund, S., Nicholson, G., Haag, H., Jung, O., Zahner, H. and Jung, G. (1993) Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. Biometals 6, 185–192.
[11] Dale, S.E., Doherty-Kirby, A., Lajoie, G. and Heinrichs, D.E. (2004) Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. Infect. Immun. 72, 29–37.
[12] Dale, S.E., Sebulsky, M.T. and Heinrichs, D.E. (2004) Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*. J. Bacteriol. 186, 8356–8362.
[13] Beasley, F.C., Vinés, E.D., Grigg, J.C., Zheng, Q., Liu, S., Lajoie, G.A., Murphy, M.E.P. and Heinrichs, D.E. (2009) Characterization of staphyloferrin A biosynthetic and transport mutants in *Staphylococcus aureus*. Mol. Microbiol. 72, 947–963.
[14] Cotton, J.L., Tao, J. and Balibar, C.J. (2009) Identification and characterization of the *Staphylococcus aureus* gene cluster coding for staphyloferrin A. Biochemistry 48, 1025–1035.
[15] Grigg, J.C., Cheung, J., Heinrichs, D.E. and Murphy, M.E. (2010) Specificity of staphyloferrin B recognition by the SirA receptor from *Staphylococcus aureus*. J. Biol. Chem. 285, 34579–34588.
[16] Grigg, J.C., Cooper, J.D., Cheung, J., Heinrichs, D.E. and Murphy, M.E. (2010) The *Staphylococcus aureus* siderophore receptor HtsA undergoes localized conformational changes to enclose staphyloferrin A in an arginine-rich binding pocket. J. Biol. Chem. 285, 11162–11171.
[17] Cheung, J., Beasley, F.C., Liu, S., Lajoie, G.A. and Heinrichs, D.E. (2009) Molecular characterization of staphyloferrin B biosynthesis in *Staphylococcus aureus*. Mol. Microbiol. 74, 594–608.
[18] Sebulsky, M.T., Speziali, C.D., Shilton, B.H., Edgell, D.R. and Heinrichs, D.E. (2004) FhuD1, a ferric hydroxamate-binding lipoprotein in *Staphylococcus aureus*: a case of gene duplication and lateral transfer. J. Biol. Chem. 279, 53152–53159.
[19] Bae, T. and Schneewind, O. (2006) Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid 55, 58–63.
[20] Lee, C.Y. and Iandolo, J.J. (1986) Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene. J. Bacteriol. 166, 385–391.
[21] Corrigan, R.M. and Foster, T.J. (2009) An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. Plasmid 61, 126–129.
[22] Sebulsky, M.T., Hohnstein, D., Hunter, M.D. and Heinrichs, D.E. (2000) Identification and characterization of a membrane permease involved in iron-hydroxamate transport in *Staphylococcus aureus*. J. Bacteriol. 182, 4394–4400.
[23] Sheldon, J.R., Marolda, C.L. and Heinrichs, D.E. (2014) TCA cycle activity in *Staphylococcus aureus* is essential for iron-regulated synthesis of staphyloferrin A, but not staphyloferrin B: the benefit of a second citrate synthase. Mol. Microbiol. 92, 824–839.
[24] Mesak, L.R., Yim, G. and Davies, J. (2009) Improved lux reporters for use in *Staphylococcus aureus*. Plasmid 61, 182–187.
[25] Paulsen, I.T., Brown, M.H. and Skurray, R.A. (1996) Proton-dependent multidrug efflux systems. Microbiol. Rev. 60, 575–608.
[26] Nishino, K. and Yamaguchi, A. (2001) Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J. Bacteriol. 183, 5803–5812.
[27] Naroditskaya, V., Schlosser, M.J., Fang, N.Y. and Lewis, K. (1993) An *E. coli* Gene *emrD* is involved in adaptation to low energy shock. Biochem. Biophys. Res. Commun. 196, 803–809.
[28] Deng, X., Sun, F., Ji, Q., Liang, H., Missiakas, D., Lan, L. and He, C. (2012) Expression of multidrug resistance efflux pump gene *norA* is iron responsive in *Staphylococcus aureus*. J. Bacteriol. 194, 1753–1762.
[29] Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105–132.
[30] Kreiswirth, B.N., Löfdahl, S., Betley, M.J., O'Reilly, M., Schlievert, P.M., Bergdoll, M.S. and Novick, R.P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305, 709–712.
[31] Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J. and Schlievert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*. J. Bacteriol. 170, 4365–4372.