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Fur is required for the activation of virulence gene expression through the induction of the *sae* regulatory system in *Staphylococcus aureus*

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Abstract

Our previous studies showed that both Sae and Fur are required for the induction of *eap* and *emp* expression in low iron. In this study, we show that expression of *sae* is also iron-regulated, as *sae* expression is activated by Fur in low iron. We also demonstrate that both Fur and Sae are required for full induction of the oxidative stress response and expression of non-covalently bound surface proteins in low-iron growth conditions. In addition, Sae is required for the induced expression of the important virulence factors *isdA* and *isdB* in low iron. Our studies also indicate that Fur is required for the induced expression of the global regulators Agr and Rot in low iron and a number of extracellular virulence factors such as the haemolysins which are also Sae- and Agr-regulated. Hence, we show that Fur is central to a complex regulatory network that is required for the induced expression of a number of important *S. aureus* virulence determinants in low iron.

Keywords

Staphylococcus aureus; Iron regulation; Fur; Sae; Agr

Introduction

S. aureus is a highly adaptable organism causing a wide range of infections both in the hospital setting and more recently in the community (Diep and Otto, 2008). Its ability to cause such a range of disease is thought to be due to its capacity to produce a variety of virulence factors (Archer, 1998). Expression of these gene products is costly to the cell, and constant changes in the microenvironment mean that production of these factors has to be tightly regulated so that they are expressed only when required. This is achieved by a

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complex regulatory network which includes the 2 component regulators SaeRS and AgrAC (Goerke et al., 2001).

A major environmental stress encountered by bacteria during infection is severe iron limitation. Iron is an essential nutrient for various key metabolic processes and so its acquisition is vital for survival. However, excess iron is toxic and therefore free-iron levels are limited in mammalian body fluids. *S. aureus* has evolved to use the low availability of iron in vivo as a major environmental signal to trigger enhanced expression of some virulence determinants, including several iron acquisition mechanisms (Dale et al., 2004; Morrissey et al., 2000, 2002), adhesion to host cells (Clarke et al., 2007, 2009), and biofilm formation (Johnson et al., 2005, 2008).

The global regulator Fur mediates iron-responsive gene regulation in many bacteria, including *S. aureus* (Horsburgh et al., 2001; Xiong et al., 2000). Fur was originally identified as an iron-dependent repressor of genes involved in the acquisition of iron (Litwin and Calderwood, 1993). However, it has recently become apparent that *S. aureus* Fur not only acts as an iron-dependent repressor, but can also act positively, inducing gene expression in both low- and high-iron conditions (Horsburgh et al., 2001; Johnson et al., 2008; Morrissey et al., 2004). Our previous studies demonstrated that biofilm formation, an important virulence determinant of *S. aureus*, is induced in low-iron growth conditions (Johnson et al., 2005). Low-iron induced biofilm formation is dependent on the secreted, non-covalently attached cell surface proteins Eap and Emp which are positively regulated in low-iron conditions by Fur (Johnson et al., 2008). Eap and Emp are important virulence factors implicated in a number of aspects of *S. aureus* pathogenesis as well as biofilm formation. Both proteins promote adhesion to a broad spectrum of host proteins (Hussain et al., 2001; Palma et al., 1999). Eap is also involved in inhibition of wound healing (Athanasopoulous et al., 2006), evasion of the host immune system (Chavakis et al., 2002, 2005), and promotion of bacterial internalisation into eukaryotic cells (Haggar et al., 2003), whilst Emp is also associated with endovascular disease (Chavakis et al., 2005). Therefore Fur is essential for the induction of these important virulence determinants in low-iron conditions reflective of growth conditions in vivo. However, the mechanisms involved in the positive Fur regulation of *eap* and *emp* have not yet been defined.

The expression of Eap and Emp is also dependent on the 2 component regulators AgrAC and SaeRS (Harraghy et al., 2005, Johnson et al., 2008). The Agr quorum sensing system is responsible for repression of cell surface proteins such as adhesins and for induced expression of exoproteins in post-exponential phase (Traber et al., 2008). The Sae regulatory system is important for expression of several toxins and immune evasion proteins (Giruado et al., 1997, Voyich et al., 2009) and is important for the response to alpha defensins and oxidative stress, which is required for neutrophil survival (Geiger et al., 2008, Voyich et al., 2005). The *sae* operon consists of 4 open reading frames (Fig. 1A), *saeP*, *saeQ*, *saeR*, and *saeS* (Novick and Jiang, 2003; Steinhuber et al., 2003; Goerke et al., 2005) in which *saeS* encodes the receptor kinase and *saeR* the response regulator (Giruado et al., 1997). The operon is auto-regulated (Novick and Jiang, 2003) and is responsive to a range of signals including SarA, SigB, high salt, hydrogen peroxide, and Agr in some strains (Adhikari and Novick, 2008; Geiger et al., 2008; Novick and Jiang, 2003; Steinhuber et al., 2003).

In this study, we demonstrate for the first time that in low-iron conditions there is a complex overlapping regulatory network involving Sae, Agr and the iron-dependent global regulator Fur. Moreover, we show that Fur is essential for the activation of virulence gene expression through induction of the *sae* regulatory system.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. Strains for biofilm assays and for protein and RNA extraction were cultured in iron-restricted conditions in CRPMI (Morrissey et al., 2000). Strains were plated out fresh from frozen stocks onto 6% defibrinated horse blood agar for each experiment. All cultures were incubated statically for 16 h at 37°C in 5% CO₂ in air. Where indicated the medium was supplemented with 50 µM Fe₂(SO₄)₃. 0.003% H₂O₂ (vol/vol) was added to Tryptic soy broth (TSB) to achieve oxidative stress. Media were supplemented with the antibiotics tetracycline (10 µg/ml) or erythromycin (10 µg/ml) where required.

Transduction of mutations into *S. aureus*

The *fur* mutation was transduced from the *fur* mutant of *S. aureus* 8325-4 (Horsburgh et al., 2001) to SH1000 (Horsburgh et al., 2002) using phage 80α, and the mutation was confirmed by PCR using the *fur* specific primers FurAF and FurAR (Table 2). The *sae/fur* double strain was constructed by transduction of the Newman *fur* mutant into Newman *sae::Tn917* strain (Goerke et al., 2001) as described previously (Johnson et al., 2009). Colonies containing the relevant mutation were confirmed by PCR using primers Fur1F, Fur1R, SaeRF, Sae flankR (Table 2).

RNA extraction for Northern blotting

RNA was extracted from exponential (6 h) *S. aureus* cultures in CRPMI and analysed by northern blot as previously described (Johnson et al., 2008). Transcripts were evaluated using ImageJ 1.41 software downloaded from <http://rsbweb.nih.gov/ij/>.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell wall and SDS surface proteins were extracted from *S. aureus* cells grown for 24 h in 10 ml of CRPMI as previously described (Hussain et al., 2001), except that dialysis to remove SDS was omitted (Johnson et al., 2008), and an equal volume of 2×Laemmli sample buffer (Laemmli, 1970) was added to cell suspensions prior to boiling for 3 min and separating by SDS–10% polyacrylamide gel electrophoresis (PAGE) as previously described (Johnson et al., 2008).

MALDI-TOF mass spectroscopy analysis

Bands excised from 1D-SDS polyacrylamide gels were subjected to in-gel trypsin digestion and analysis as per standard procedures (Speicher et al., 2000) within the PNACL Proteomics Facility, University of Leicester, as described previously (Johnson et al., 2008).

Hydrogen peroxide growth assays

Bacteria were grown overnight in TSB at 37°C with shaking in air, diluted to an optical density at 600 nm of 0.05 in fresh TSB with appropriate antibiotics, and then split into duplicate cultures and incubated with shaking at 37°C for 4 h. The OD₆₀₀ was measured and 0.003% H₂O₂ (vol/vol) was added to one of the duplicates; both were then incubated with shaking at 37°C, and the OD₆₀₀ was measured 2 h after addition of H₂O₂.

Transcriptional profiling

S. aureus SH1000 and its Δfur mutant strain were inoculated on TSB agar and then transferred to 5 ml of defined medium (DM) (Townsend and Wilkinson., 1992) and grown at 37°C for 18 h with aeration. Each strain was then diluted 1:10 into chelex-treated (Bio-

Rad) DM without iron and allowed to grow in acid-washed glassware to deplete the organisms' internal iron pools. This was used as starter culture which was diluted 20× in fresh chelex-treated DM without or with iron (100 µM FeCl₃) and incubated with aeration at 37°C. Growth was monitored at OD₆₀₀ in a spectrophotometer. Samples for transcriptional profiling experiments were taken at early log-phase (OD₆₀₀ 0.6-0.7). *S. aureus* SH1000 wild type served as the control, whereas the *S. aureus* SH1000 *fur* mutant was the test sample. The DNA microarray experiments were performed using 3 independently cultured sample sets to isolate the RNA, and each set was run independently to generate triplicate data. RNA extraction, cDNA synthesis, labelling, and array hybridisation were carried out as described previously (Muthaiyan et al., 2008). The denatured cDNA was applied to a *S. aureus* genome microarray version 5.0 DNA chip (Pathogen Functional Genomic Resource Center – The Institute of Genomics Research, PFGRC-TIGR <http://pfgrc.jcvi.org>) under a glass slide, and hybridization was carried out overnight at 42°C. After hybridization, the slides were washed at room temperature in wash buffer I (1× SSC, 0.2% SDS) twice for 10 min, in wash buffer II (0.1× SSC, 0.1% SDS) once for 10 min, and in wash buffer III (0.1× SSC) twice for 10 min. The slides were dried by centrifugation at 500 rpm for 5 min.

Data analysis

Hybridization signals were scanned using Axon 4000B scanner with Acuity 4.0 software. Scans were analysed using TIGR-Spotfinder software, and the data set was normalized by applying the LOWESS algorithm using TIGR-MIDAS software (www.tigr.org/software/). Significant changes of gene expression were identified with significance analysis of microarrays (SAM) software using one class mode. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. A “q value” assigned to each gene corresponds to the lowest false discovery rate at which the gene is called significant (43). Three independent cultures were used to prepare RNA.

Quantitative real-time PCR analysis

Prior to RNA isolation SH1000 and Δfur were grown in defined media in the presence and absence of iron under the same conditions as used for the transcriptional profiling. RNA was isolated using the RNeasy Protect Bacteria Mini Kit (Qiagen) and DNA removed from the samples by DNase I treatment (Qiagen) according to manufacturer's instructions. The purified RNA was quantified using the nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and the integrity assessed by electrophoresis. 0.5 µg of RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to manufacturer's instructions. qRT-PCR was performed using the *Power* SYBR Green PCR Master Mix (Applied Biosystems) in a 7300 Fast System (Applied Biosystems) according to manufacturer's instructions. The relative levels of gene expression in the treated cells and the non-treated controls were calculated by relative quantification using *16S rRNA* gene as the endogenous reference. All samples were amplified in triplicate, and the data analysis was carried out using the 7300 Fast System Software (Applied Biosystems).

Results

Expression of *sae* in low iron is dependent on Fur and Agr

Multiple regulators, including Fur, Sae, and Agr, are involved in the low-iron-mediated induction of *eap* and *emp*; however, it is not clear how these regulators interact or how Fur induces the expression of *eap* and *emp*. It is possible that iron/Fur regulation of *eap* and *emp* is indirect and mediated via regulation of the gene encoding the positive regulator Sae. Therefore, we investigated the iron-responsive regulation of *sae* transcription. Total RNA was extracted from Newman wild type and the Newman *fur* mutant growing exponentially

in CRPMI with or without added iron. Expression of the *sae* operon is complex as there are 2 promoters and possible post-transcriptional modification resulting in 4 different transcripts designated A–D (Fig. 1A) (Adhikari and Novick, 2008;Giraud et al., 2003). Therefore Northern blot analysis was carried out using a combination of 2 *sae*-specific probes, *saeP* which detects transcripts D and C, and *saeR* which detects transcripts A, B, and C (Adhikari and Novick, 2008). In Newman wild type, all 4 *sae* transcripts were decreased by the addition of iron (Fig. 1B) indicating that this important global virulence gene regulator is iron-regulated. However, in the *fur* mutant, no *sae* transcripts were detected suggesting that Fur is essential for the activation of *sae* transcription. Therefore, it appears that Fur acts as an activator and not a repressor of *sae* expression suggesting that there is another iron-dependent repressor in *S. aureus* other than Fur. This is the same phenotype as observed previously for low-iron and Fur-mediated regulation of *eap* and *emp* transcription (Johnson et al., 2008). These observed differences are not due to growth effects as although the addition of iron improved growth very slightly compared to iron-restricted medium, there was no overall difference in growth between the *S. aureus* strains in iron-restricted or iron-replete CRPMI (data not shown). Therefore, it appears that low-iron and Fur regulation of *eap* and *emp* transcription is indirect and is mediated through Fur-dependent regulation of *sae* which positively regulates *eap* and *emp* expression.

Northern blot analysis also showed that *sae* transcription in the *agr* mutant in low-iron medium was decreased compared to the wild type (Fig. 1C). No *sae* operon transcripts were detected in the *agr* mutant, which corresponds to the lack of exoprotein expression seen in our previous studies (Johnson et al., 2008). However, this observation is in contrast to previous studies which show that *S. aureus* Newman *sae* is not regulated by Agr because of mutation in the gene encoding the auto-activator SaeS (Adhikari and Novick, 2008;Geiger et al., 2008). DNA sequencing showed that the mutation in the *saeS* open reading frame was also present in the Newman isolate used in this study, which correlates with the observed increase in expression of many Sae-regulated proteins such as Eap and Emp also seen in Newman in other studies (Harraghy et al., 2005;Johnson et al., 2008). Thus, in low-iron Newman *sae* expression is induced by Agr despite the amino acid substitution in SaeS. Therefore, these results demonstrate that under low-iron growth conditions *sae* expression is dependent on Fur and Agr.

***S. aureus fur* is negatively regulated by Agr and Sae**

We further investigated the relationship between these regulators by determining whether *fur* is regulated by Sae or Agr. Total RNA was extracted from Newman wild type and its isogenic Δfur , Δagr , and Δsae mutant strains growing exponentially in CRPMI with or without the addition of iron. Northern blot analysis using a *fur*-specific probe revealed 2 transcripts of approximately 1.3 and 0.5 kb (Fig. 2B), suggesting that *fur* is cotranscribed with the downstream gene *xerD* (Fig. 2A). Transcript levels in the wild type were significantly decreased by the addition of iron, indicating that *fur* expression is regulated by iron. Northern blot analysis using a *xerD*-specific probe detected a single iron-repressed transcript in Newman of approximately 1.3 kb, confirming that *fur* and *xerD* are cotranscribed (Fig. 2C). In the *fur* mutant, there was a single larger *fur/xerD* transcript of approximately 2.4 kb corresponding to the few remaining bases of the *fur* gene, the tetracycline cassette used in the construction of the *fur* mutant (Horsburgh et al., 2001), and the *xerD* gene. The presence of this transcript confirms that the *fur* mutation does not affect *xerD* expression which is supported by the fact that *xerD* mutation is lethal in *S. aureus* (Chalker et al., 2000). The level of $\Delta fur::tet/xerD$ transcript was increased compared to the wild-type *fur/xerD* transcript, but was still repressed in high iron (Fig. 2C), which suggests that *fur* is auto-repressed but that, interestingly like *sae* (Fig. 1B), the *fur/xerD* operon is also iron-regulated independently of *fur*.

Northern blot analysis also demonstrated that the 0.5-kb *fur* transcript is regulated by Agr and Sae (Fig. 2B). Densitometry analysis showed that the 0.5-kb transcript was increased 4-fold in the *agr* mutant and 2-fold (± 0.216) in the *sae* mutant, indicating that Agr and to a lesser extent Sae repress Fur transcription. Thus, there appears to be a complex, regulatory feedback system encompassing Fur, Sae, and Agr.

The Fur and Sae regulons overlap, and both regulators are required for oxidative stress resistance

To determine the global effect of combining the *sae* and *fur* mutations, proteins were prepared from cell wall and SDS surface extracts from *S. aureus* Newman wild type and the isogenic *sae*, *fur*, and *sae/fur* mutants grown in CRPMI with and without iron. In the cell wall extracts, 3 major proteins were shown to be induced in low iron; these were identified by MALDI-TOF mass spectroscopy as IsdB (FrpB), IsdA (FrpA), and Eap (Fig. 3A). IsdA and IsdB are involved in haem uptake and have characteristic LPXTG motifs that covalently anchor them to the cell surface (Mazmanian et al., 2003), whereas Eap is non-covalently attached to the cell surface (McGavin et al., 1993). The *isdA* and *isdB* genes contain Fur-binding consensus sequences in their promoters and are classically regulated by Fur (Morrissey et al., 2002), whereas *eap* transcription is positively regulated by Fur in low iron but has no obvious Fur-binding consensus sequence in its promoter (Johnson et al., 2008). This Fur regulation is confirmed by the observation that in the *fur* mutant IsdA and IsdB are constitutively expressed, whereas there is a decrease in Eap levels (Fig. 3A). In the *sae* and *fur/sae* mutants, no Eap protein could be detected, therefore showing that *eap* is positively regulated by both Sae and Fur as seen previously (Johnson et al., 2008).

Interestingly *isdA* and *isdB* expression is also regulated by Sae at the transcriptional level. Northern blot analysis of total RNA extracted from Newman wild type and Δ *sae* mutant strains growing exponentially in CRPMI showed that both *isdA* and *isdB* expression is decreased in Newman *sae* compared to Newman wild type (Fig. 3C). Therefore, these results show that components of the Fur regulon are positively regulated by Sae.

Overlap of the Fur and Sae regulons is further demonstrated by the SDS surface extracts as there are very few proteins expressed in low-iron growth conditions in the *sae fur* double mutant compared to the single *sae* and *fur* mutants (Fig. 3B). There is also differential regulation of a number of SDS surface extract proteins in the *sae fur* mutant compared to the single mutants (Fig. 3B). As shown previously (Johnson et al., 2008), Eap levels are lower in the SDS surface protein extracts of the *fur* mutant and absent in those of the *sae* and *sae fur* mutants (Fig. 3B). These results therefore suggest that the Sae and Fur regulons overlap and have a significant effect on global protein expression.

Both Fur and Sae have been implicated in oxidative stress resistance (Geiger et al., 2008; Horsburgh et al., 2001; Morrissey et al., 2004), therefore to further investigate the combined effect of these regulators H₂O₂ was added to one of duplicate cultures of *S. aureus* Newman wild type and isogenic *fur*, *sae*, or *fur/sae* mutant strains growing exponentially in TSB medium. A rich medium under aeration was used to ensure that quantifiable levels of oxidative stress could be assayed as all strains were hypersensitive to H₂O₂ in the nutrient-deficient medium CRPMI, and therefore reproducible results could not be obtained. Addition of H₂O₂ decreased the growth of all strains except the *sae* mutant which showed a 1% decrease in growth ($P=0.003$) compared to untreated control. Newman wild type demonstrated a 10% decrease in growth, whereas the *fur* mutant strain showed a 35% decrease in growth after the addition of H₂O₂ ($P=0.0001$). However, the *fur sae* double mutant was significantly more sensitive to H₂O₂ than any of the other strains as it showed a 70% decrease in growth ($P=0.0001$). Therefore, both Sae and Fur are required for full induction of the *S. aureus* oxidative stress response.

***S. aureus* Fur is required for the expression of a number of Sae- and Agr-regulated virulence factors in low iron**

Previous transcriptional profiling has shown that Sae is a global regulator affecting the expression of a vast array of genes (Voyich et al., 2005, 2008), however, no transcriptional profiling studies have been published to date detailing the role of Fur in global gene expression in *S. aureus*. To determine the role of Fur in *S. aureus* gene expression in iron-replete conditions SH1000 wild type and *fur* mutant strains were grown in DM with 100 μ M FeCl₃. SH1000 was used in the microarray studies to begin to address iron and Fur global regulation conservation between strains as many previous global iron regulation studies have been done in Newman. Total RNA was extracted from exponentially growing staphylococci. cDNA samples were hybridised with the PFGRC *S. aureus* genomic microarray. After applying statistical evidence with a ≥ 2.0 -fold difference as the threshold change and a *P* value less than 0.05, microarray analysis showed that 134 genes were up-regulated and 76 genes were down-regulated in the *fur* mutant compared to the wild type in iron-replete conditions (Table S1).

Fur is normally an active repressor in the presence of iron and so genes de-repressed in the *fur* mutant are candidate members of the classical Fur regulon. Therefore, not surprisingly many known Fur-regulated genes such as the iron acquisition systems and siderophore biosynthesis genes were up-regulated in the *fur* mutant compared to the wild type (Table 3) (Delany et al., 2004; Morrissey et al., 2000). Conversely, the ferritin (*ftnA*) and catalase (*katA*) genes were repressed in the *fur* mutant compared to the wild type, which was also expected as these genes are known to be positively regulated by Fur (Horsburgh et al., 2001; Morrissey et al., 2004). These results have been validated by qRT-PCR (Table 4) and are in agreement with the literature and our previous studies (Morrissey et al., 2000, 2004), and therefore these microarray results are representative of the *S. aureus* Fur regulon in iron-replete conditions. Other genes de-repressed in the *fur* mutant in iron-replete conditions include the capsule synthesis genes *cap5D*, *cap5G*, and *capH* and the purine ribonucleotide biosynthesis genes *purE*, *K*, *Q*, *C*, and *L* (Table S1), which is consistent with previous studies demonstrating capsule synthesis being iron-regulated (Lee et al., 1993). Genes down-regulated in the *fur* mutant in iron-replete conditions include a number of regulators such as *srrA* and SACOL2531, which encodes a putative transcriptional regulator of the MarR family (Table S1). Hence *S. aureus* Fur has a global effect on iron-regulated gene expression which encompasses many aspects of staphylococcal physiology and virulence in addition to the acquisition and storage of iron.

To investigate the global role of Fur in *S. aureus* gene expression in low-iron conditions, SH1000 wild type and *fur* mutant strains were grown in chelex-treated DM, and microarray analysis was conducted as described above. This transcriptional profiling showed that 35 genes were up-regulated and 90 genes were down-regulated in the SH1000 *fur* mutant compared to the wild type in low-iron conditions (Tables 5, 6, and S2). The transcriptional profiling confirmed our previous results (Johnson et al., 2008; Fig. 1B) and demonstrated that Fur is required for induced expression of *sae* and *eap* in SH1000 as well as Newman, as the expression of both genes was decreased in the SH1000 *fur* mutant compared to the wild type (Tables 5 and 6). Therefore, positive regulation of *sae* by Fur occurs in *S. aureus* in different media and strains independently of the *saeS* mutation. Therefore it is likely that it is a general regulatory response. Furthermore, transcriptional profiling showed that the expression of a number of other Sae-regulated genes was also repressed in the *fur* mutant compared to the wild type including the staphylococcal nuclease (*nuc*), glycerol ester hydrolase (*geh*), and the α , β , and γ -haemolysin genes (Table 5). Therefore, Fur is required for the induced transcription of a number of important virulence factors in low-iron growth conditions many of which are also Sae-regulated, a finding that is also in agreement with our proteomic data in Newman (Fig. 3A).

However, the transcriptional profiling revealed that Sae is not the only *S. aureus* global regulator which requires Fur for induced expression in low iron, as the transcript levels of *agrA* and *rot* (repressor of toxins) were also decreased in the *fur* mutant in low iron compared to the wild type (Table 6). Furthermore, a number of the virulence factors shown to be positively regulated by Fur are also regulated by Agr as well as Sae (Table 5). Therefore, in low-iron growth conditions *S. aureus* Fur is required for the expression of 3 major global virulence gene regulators, Sae, Agr, and Rot, and hence Fur is important for the expression of a diverse range of important virulence determinants (Fig. 4).

Discussion

In this study, we show for the first time that Fur is central to a complex overlapping regulatory network, also involving the global regulators Sae and Agr, that is required for the induced expression of a number of important *S. aureus* virulence determinants (Fig. 4). Our previous studies showed that both Sae and Fur are required for the induction of *eap* and *emp* expression in low iron. In this study, we show that expression of *sae* is also iron-regulated, as *sae* is induced by Fur in low iron. We also demonstrate that both Fur and Sae are required for full induction of the oxidative stress response and expression of non-covalently bound surface proteins in low-iron growth conditions. Interestingly, Fur and Sae show both complementary and differential regulation as both Fur and Sae positively regulate *eap* and *emp* expression in low iron, whereas Fur negatively and Sae positively regulates *isdA* and *isdB* transcription. Furthermore, our microarray studies indicate that Fur may also be required for the induced expression of the Agr and Rot regulators in low iron as well as the expression of a number of extracellular virulence factors such as the haemolysins which are also Sae-, Agr-, and Rot-regulated. Hence, Fur is fundamental to *S. aureus* virulence gene expression.

The Sae regulatory system is essential for biofilm formation (Johnson et al., 2008), the adaptive response to alpha defensins and oxidative stress (Geiger et al., 2008), innate immune evasion and staphylococcal survival in neutrophils (Voyich et al., 2005, 2009). Although *sae* is expressed in the presence of high concentrations of iron and in iron-rich, complex growth conditions such as TSB, it is at a much lower level than in low-iron, nutrient-poor media reflective of the in vivo environment. This result is consistent with our previous studies showing that Eap and Emp expression levels are significantly higher when *S. aureus* Newman is grown in low-iron medium, compared to their expression in iron-replete, nutrient-rich media such as TSB (Johnson et al., 2008). Surprisingly, there is differential regulation of the *sae* transcripts C and D even though D is processed from transcript C and therefore you would expect to see the same level of regulation. However, it is possible that after processing the stability of transcript C is reduced compared to transcript D resulting in a lower observed level of transcript C. This is the first time that *sae* transcription has been shown to be induced in low-iron conditions and to require Fur for this induced expression. Fur regulation of *sae* seems to have a major effect on global gene expression as Fur not only induces expression of *eap* and *emp*, but, as our proteomics and microarray studies show, Fur and Sae are cooperatively involved in the induction of many exoproteins including the haemolysins, nuclease, lipase, and the IgG-binding protein SBI.

The importance of both *sae* and *fur* in virulence has been established previously (Goerke et al., 2001; Horsburgh et al., 2001; Nygaard et al., 2010; Voyich et al., 2009; Xiong et al., 2000), but the link between these 2 regulators had not been established until now. Previous transcriptional profiling of *S. aureus* grown in vitro in low iron (Allard et al., 2006) and proteomics analysis of cytoplasmic proteins (Freidman et al., 2006) indicated that iron and Fur have a global effect on *S. aureus* gene expression, although an effect on Sae was not observed. However, the role of Fur in the transcriptional activation of many of the

exoprotein virulence determinants or essential global virulence gene regulators in low iron had not been shown previously in *S. aureus*, or to our knowledge any other bacteria. This study is the first to show that the *S. aureus* Fur is not just involved in the transcriptional regulation of iron acquisition and storage but is actually essential for the transcription of many virulence genes in low-iron growth conditions possibly mediated through the regulation of important global regulators.

Our previous studies had only shown Fur-mediated low-iron induction of gene expression in *S. aureus* Newman. However, this study shows the same regulation occurring in a different *S. aureus* strain, SH1000, as *eap*, *emp*, and *sae* are all positively regulated by Fur in low iron in Newman and SH1000, hence Fur positive regulation in low iron appears to be a general staphylococcal regulatory. However, how Fur activates transcription in low iron is not yet known. Fur could be directly affecting *sae* expression which in turn would affect the expression of the exoproteins such as *eap* and *emp*. However, this does not explain the Fur-mediated regulation of *sae* itself. It is possible that *S. aureus* Fur activates transcription through direct binding of target promoters as in *Neisseria meningitidis* (Delany et al., 2004), although there are no obvious Fur box consensus sequences in any of the positively Fur-regulated promoters. Alternatively, Fur could act indirectly by repressing the expression of another protein regulator or could be involved in post-transcriptional regulation possibly mediated by small, non-coding regulatory RNAs (Majdalani et al., 2005). Regulatory small RNAs have been identified in *S. aureus* but their roles in gene expression have yet to be fully elucidated (Pichon and Felden, 2005). Fur normally binds DNA when complexed with iron, but we show here that the *S. aureus* Fur induces expression in low iron. Therefore, there is a possibility that the *S. aureus* iron-free form of Fur can bind DNA and either repress or activate transcription of downstream regulators as seen in *Helicobacter pylori* (Bereswill et al., 2000).

The transcriptional profiling suggests that *S. aureus* Fur positively regulates the *agr* and *rot* transcriptional regulators as well as many Agr- and Rot-regulated exoprotein genes. In addition, transcriptional analysis shows that Agr appears to positively regulate *sae* transcription and negatively regulate *fur* transcription in low iron. This means that there is a complex feedback loop and that the transcription of all 3 major regulators is closely linked, with Fur playing a central role, although how this regulation occurs is not yet clear (Fig. 4). Previous studies have indicated that in *S. aureus* Newman *sae* expression is not regulated by Agr due to an amino acid substitution in the sensor histidine kinase SaeS which results in *sae* hyperactivation (Adhikari and Novick, 2008; Geiger et al., 2008). However, we show here that *sae* is clearly regulated by Agr, Fur, and iron in *S. aureus* Newman despite the amino acid substitution in SaeS. This could be due to the different growth medium used in this study and the possible overriding effects of iron. Alternatively our results suggest that Agr, Fur, and iron-mediated regulation of *sae* is not via SaeS auto-activation, but is mediated by another mechanism possibly acting directly via the *sae* operon promoter sequences.

This study indicates that iron concentrations and Fur play an important role in global virulence gene regulation. The low iron concentrations found in vivo can be used as a signal to up-regulate expression of virulence genes. Therefore, it is not surprising that the expression of virulence gene regulators such as Sae is also induced in response to low iron. Sae has been shown to be important for bloodstream infections and staphylococcal survival after neutrophil phagocytosis (Voyich et al., 2005, 2009). Therefore, the low iron concentrations encountered in these environments could be involved in the up-regulation of *sae* and the Sae-regulated genes required for *S. aureus* survival in neutrophils. Positive regulation of *sae* by Fur suggests that Fur is also required for innate immune evasion and intracellular survival in *S. aureus*. This hypothesis is also supported by the fact that this

study shows that both Sae and Fur are required for maximal induction of the oxidative stress response which will be required for intracellular survival in neutrophils. It is interesting that the *sae* mutant is more resistant to oxidative stress than the wild type, and yet the *sae/fur* double is clearly even more sensitive than the single *fur* mutant. It is not clear why this is the case as the actual role of Sae in oxidative stress resistance has not yet been determined. Expression of *fur* is de-repressed in the *sae* mutant and therefore the oxidative stress response may be up-regulated via Fur in the *sae* mutant. Nevertheless both regulators are clearly required for the full response to occur. Our results suggest that Sae, and therefore possibly Fur, may also be essential for staphylococcal survival in the blood and neutrophils as it is required for the induced expression of *isdA* and *isdB* in low iron. A substantial level of IsdA and IsdB polypeptide was still observed in the *sae* mutant extracts although noticeably lower levels of transcripts were detected. This indicates that transcription occurred allowing some translation, but the mRNA was quickly degraded, suggesting that Sae may be involved in post-transcriptional regulation of *isdA* and *isdB* expression. IsdA and IsdB have been shown to be important for iron uptake from haem and haemoglobin (Mazamania et al., 2003) as well as for survival in neutrophils (Voyich et al., 2005). Positive regulation of these important virulence determinants by Sae has not been shown previously, and therefore this data adds another dimension to the role of Sae in pathogenesis. Furthermore, the Sae- and Fur-regulated proteins IsdA, IsdB, Eap, and Emp have been shown to be required for abscess formation and persistence following intravenous infection of mice, suggesting that Fur may also play a role in persistence (Cheng et al., 2009). Therefore our results suggest that Fur has a fundamental role in *S. aureus* virulence as Fur is required for the induced expression in low iron of *sae*, *agr*, and a number of secreted proteins all of which are essential for virulence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (A) Structure of the *sae* operon, showing positions of promoters (P_1 and P_3) and *sae* operon transcripts (A-D). (B and C) Northern blot analysis of total RNA prepared from *S. aureus* Newman and its isogenic *fur* and *agr* mutants growing exponentially in CRPMI (–) or CRPMI with 50 μ M $\text{Fe}_2(\text{SO}_4)_3$ (+). Ten μ g of total RNA were resolved by agarose gel electrophoresis and hybridised with both the *saeR* and *saeP* DNA probes. The blot was then stripped and rehybridised with the 16S rRNA control probe.

Fig. 2.

Northern blot analysis of total RNA prepared from *S. aureus* Newman and its isogenic *sae*, *agr*, and *fur* mutants growing exponentially in CRPMI (–) or CRPMI with 50 μ M $\text{Fe}_2(\text{SO}_4)_3$ (+). Ten μ g of total RNA were resolved by agarose gel electrophoresis and hybridised with (B) *fur* and (C) *xerD* DNA probes. The blots were then stripped and rehybridised with the 16S rRNA control probe. (A) Genetic organisation of *fur-xerD* operon.

Fig. 3.

(A) Cell wall. (B) SDS surface extract obtained from *S. aureus* Newman and its isogenic mutants *sae*, *fur*, and *sae/fur* grown for 16 h in iron-restricted CRPMI (–) or CRPMI with 50 μM $\text{Fe}_2(\text{SO}_4)_3$ (+) added to give iron-replete growth conditions. All lanes are equivalently loaded. Gels presented are representative of experiments repeated at least 3 times using protein extracted from post-exponential phase cultures grown on different days with similar results observed each time. (C) Northern blot analysis of total RNA prepared from *S. aureus* Newman and its isogenic *sae* mutant growing exponentially in CRPMI (–). Ten μg of total RNA were resolved by agarose gel electrophoresis and hybridised with (C) *isdA* or *isdB* DNA probes. The blots were then stripped and rehybridised with the 16S rRNA control probe.

Fig. 4. Model of a regulatory network involving Fur, Sae, and Agr showing that Fur is a central regulator of virulence gene expression. Positive and negative regulatory pathways are indicated with either a + or -, respectively.

Table 1

Bacterial strains used in this study.

Strain	Description	Reference or source
Newman	Wild type	(Duthie and Lorenz., 1952)
Newman <i>fur</i>	Newman $\Delta fur::tet$	(Johnson et al., 2005)
Newman <i>sae</i>	Newman <i>sae</i> : : <i>Tn917</i> (AS3)	(Goerke et al., 2001)
Newman <i>sarA</i>	Newman $\Delta sarA::ermC$	(Johnson et al., 2008)
Newman <i>agr</i>	Newman $\Delta agr::tetM$	(Johnson et al., 2008)
Newman <i>fur/sae</i>	Newman $\Delta fur::tet$, <i>sae</i> : : <i>Tn917</i> (AS3)	This study
SH1000	NCTC 8325-4 with <i>rsbU</i> mutation repair	(Horsburgh et al., 2002)
8325-4 <i>fur</i>	8325-4 $\Delta fur::tet$	(Horsburgh et al., 2001)
SH1000 <i>fur</i>	SH1000 $\Delta fur::tet$	This study

Table 2

Primers used in this study

Description	Primer name	Sequence (5'–3')
<i>saeP</i> probe	saePF	GCAGCTGGTGCTGTTATCAC
	saePR	CGAAGATGACGTGAGC
<i>saeR</i> probe	saeRF	GGTGCAGATGACTATG
	saeRR	GGTATACTCGATACGACG
<i>fur</i> RNA probe	FurF	CATCTACACGACCACATTCC
	FurR	CGATTAAATCGCGTTAAGCAAC
<i>sae</i> gene sequencing	Sae Flank F	GTTTATTTCAACATTTAATAGC
	SaePR	CGAAGATGACGTGAGC
	Sae Int F	CGCAATGGTTGACTACGA
	Sae Int R	GTTTATTTCAACATTTAATAGC
	SaeSF	GGTGTTATCAATTAGAAGTC
	Sae Flank R	GAGAAGGATACCCATAAAG
<i>isdA</i> probe	isdAF	GGATGACTATATGCAACACC
	isdAR	GTTCTTGAGCAGTTTGTG
<i>isdB</i> probe	isdBF	CTGTTGAGTTCCATCTTC
	isdBR	CTACAACATGAACAAACGG
Fur mutant	FurAF	CTATCCTTTACCTTTAGCT TGGCA
	FurAR	TTGGAAGAACGATTAAATCGC
Fur mutant	Fur1F	AGGTACAGTTCACCTTAGATG
	Fur1R	CGCGCAATAGATTTAGCAG
<i>fhuA</i> RT-PCR	fhuAF	ATGAATCGTTTGCATGGA
	fhuAR	AATGATTGACGTCACCTTTGC
<i>sstC</i> RT-PCR	sstCF	TAAAGAAGAACGTTTCCATGGAC
	sstCR	CTTGAAAAGTCAATTTTTGGTGC
ftnA RT-PCR	ftnAF	ATGAATCGTTTGCATGGACA
	ftnAR	TTGACGTCACCTTGCCATCT
<i>atl</i> RT-PCR	atlF	TTAGTTACAGAGTTGTGTCATTCCA
	atlR	GGTAATCTAGATGCGCCGAATAA

Table 3Genes with differential expression in *fur* mutant compared to wild type in high iron growth conditions

Gene	Description	Fold change
Iron acquisition and storage		
<i>htsB</i>	Iron-compound binding protein	10.6
<i>htsA</i>	Iron-compound permease protein	4.0
<i>fhuD</i>	Ferrichrome-binding protein	7.9
<i>isdI</i>	Heam mono-oxygenase	5.8
<i>isdA</i>	Heam-binding protein	4.5
<i>sstC</i>	Iron transport ATP-binding protein	3.1
<i>fhuB</i>	Ferrichrome transport permease protein	2.6
<i>fhuG</i>	Ferrichrome transport permease protein	2.3
<i>htsC</i>	Heme transport system permease	2.0
<i>htsB</i>	Heme transport system permease	2.4
<i>sbnB</i>	Siderophore biosynthesis gene	17.5
<i>sbnE</i>	Siderophore biosynthesis gene	5.3
<i>sbnF</i>	Siderophore biosynthesis gene	25.9
<i>sbnG</i>	Siderophore biosynthesis gene	15.9
<i>sbnH</i>	Siderophore biosynthesis gene	23.6
<i>fntA</i>	Ferritin	-11.4
<i>katA</i>	Catalase	-2.5

Table 4

qRT-PCR results showing differentially expressed SH1000 genes in response to iron starvation

Gene	Fold value	Standard deviation
<i>fhuA</i>	2.1	0.083
<i>sstC</i>	3.1	0.009
<i>finA</i>	-1.8	0.058
<i>Atl</i>	8.2	0.088

Table 5Genes with differential expression in *fur* mutant compared to wild type in low-iron growth conditions

Gene	Description	Fold change	Positively regulated
Exoproteins/virulence factors			
<i>sbi</i>	IgG-binding protein SBI	-5.9	Sae
<i>aur</i>	Zinc metalloproteinase aureolysin	-5.2	AgrA
<i>eap</i>	Extracellular adherence protein	-4.3	Sae
<i>geh</i>	Glycerol ester hydrolase	-3.4	Sae, AgrA
<i>sspB2</i>	Cysteine protease precursor	-3.0	
<i>hla</i>	α -haemolysin	-2.4	Sae, AgrA
<i>hlgC</i>	γ -haemolysin, C component	-2.4	Sae, AgrA
<i>hly</i>	β -haemolysin	-2.1	Sae
<i>nuc</i>	Staphylococcal nuclease	-2.4	Sae
<i>atl</i>	Bifunctional autolysin	2.6	
<i>lytM</i>	Peptidoglycan hydrolase	2.6	
<i>rpoC</i>	RNA polymerase	-2.3	

Table 6Genes with differential expression in *fur* mutant compared to wild type in low-iron growth conditions

Gene	Description	Fold change
Regulators		
<i>saeR</i>	Response regulator SaeR	−2.9
<i>saeS</i>	Sensor histidine kinase SaeS	−2.3
<i>agrA</i>	Accessory gene sensor histidine kinase	−2.0
<i>rot</i>	Repressor of toxins	−2.4