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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.

Introduction

Staphylococcus 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 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 (Athanasopoulos 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

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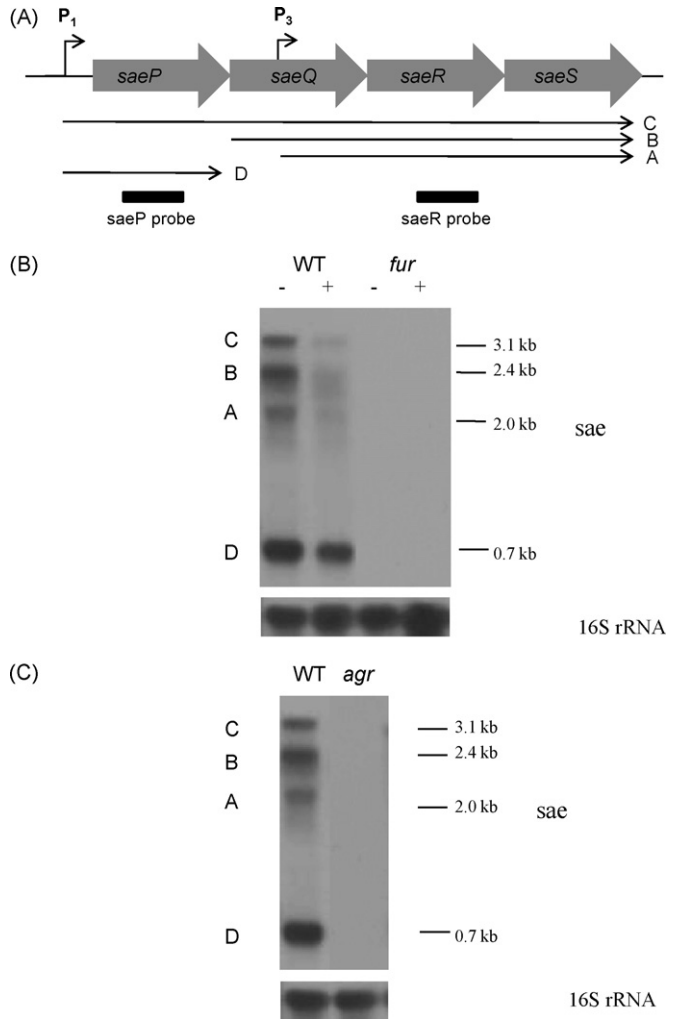

Fig. 1. (A) Structure of the *sae* operon, showing positions of promoters (P_1 and P_3) and *sae* operon transcripts (A–D). (B and C) Northern blot analysis of total RNA prepared from *S. aureus* Newman and its isogenic *fur* and *agr* mutants growing exponentially in CRPMI (−) or CRPMI with 50 μM $Fe_2(SO_4)_3$ (+). 10 μg 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.

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 two 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 (Giraudo 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 (Giraudo 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).

Table 1
Bacterial strains used in this study.

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

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% CO_2 in air. Where indicated the medium was supplemented with 50 μM $Fe_2(SO_4)_3$. 0.003% H_2O_2 (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., 2008). 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