Haemin represses the haemolytic activity of *Staphylococcus aureus* in an Sae-dependent manner

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Staphylococcus aureus is a major human pathogen and a common cause of nosocomial infections. This facultative pathogen produces a large arsenal of virulence factors, including the haemolysins, which allow the bacterium to lyse erythrocytes and thereby release large amounts of the haem-containing haemoglobin. The released haem is thought to be the main iron source of this organism during the course of infection, and is considered to be crucial for bacterial proliferation *in vivo*. High concentrations of haem and its degradation products, on the other hand, are known to be toxic for *S. aureus*, making it essential for the pathogen to tightly control haem release from red blood cells. Here we show that *S. aureus* responds to haemin by downregulating the expression of haemolysins. Subinhibitory concentrations of haemin were found to significantly reduce transcription of the haemolysin genes *hly* (encoding β -haemolysin) and *hlyA* (encoding the S-class component of γ -haemolysin), while *hly* (encoding α -haemolysin) and *RNAIII* (encoding δ -haemolysin) transcription did not appear to be affected. The presence of haemin also reduced the haemolytic potential of the supernatants of *S. aureus* LS1 cultures. Inactivation of the *sae* locus in LS1 abolished the haemin effect on the transcription of haemolysin genes, indicating that the two-component regulatory system is required for this regulatory effect. Iron limitation, on the other hand, was found to induce the expression of haemolysins, and this effect was again abolished in the *sae* mutant, indicating that *S. aureus* modulates its haemolysin production in response to iron and haem availability in an Sae-dependent manner.

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INTRODUCTION

Iron is a key element for all living organisms due to its important role in enzyme processes and transport of electrons, mainly acting as a cofactor for other proteins or enzymes (Somerville & Proctor, 2009). In mammals, free iron is scarce, perhaps to prevent pathogenic growth and protect the organism itself from this reactive element in its ionic form, Fe^{2+} (Stojiljkovic & Perkins-Balding, 2002; Touati, 2000). Thus, 99.9% of the iron in humans is located intracellularly (Reniere *et al.*, 2007), and the largest amount (approx. 75% of total iron) is complexed within the haem molecule inside erythrocytes (Stojiljkovic & Perkins-Balding, 2002).

Staphylococcus aureus is a major human pathogen and a frequent cause of life-threatening nosocomial and community-acquired infections (Diekema *et al.*, 2001) such as sepsis, endocarditis, osteomyelitis and pneumonia (Lowy, 1998). An arsenal of virulence factors enables this bacterium to initiate colonization and to facilitate persistence within the host (Horsburgh *et al.*, 2001). Among these factors, cytotoxins, and specifically haemolysins, are secreted by *S. aureus*, leading to red blood cell lysis, release of iron complexes and a local increase in the available iron concentration of up to 100-fold (Rouault, 2004). The released haemoglobin is bound by the bacterium to special receptors, and the haem subunit is released and imported into the cytoplasm via two different systems called Isd (iron-regulated surface determinant system) and Hts (haem transport system) (Reniere *et al.*, 2007).

The Isd system consists of 10 genes, expression of which is controlled by the iron-dependent ferric uptake regulator Fur (reviewed by Maresso & Schneewind, 2006). The *isd*

Abbreviations: qRT-PCR, quantitative real-time RT-PCR; RLU, relative light units.

Two supplementary tables are available with the online version of this paper.

locus encodes three cell wall-associated proteins (IsdA, B and C), one transpeptidase (SrtB), three membrane transport proteins (IsdD, E and F) and the haem oxygenase IsdG (Mazmanian *et al.*, 2003), while IsdH (synonym HraA), another cell wall-anchored factor of this system, and IsdI, a second haem oxygenase, are located elsewhere on the chromosome (Dryla *et al.*, 2003; Skaar *et al.*, 2004). IsdB and IsdH bind haemoglobin on the cell surface (Dryla *et al.*, 2003; Torres *et al.*, 2006). The haem subunit is released from this complex via an as-yet-undefined mechanism, transported through the cell wall via IsdC, and passed through the cell membrane to the cytoplasm with IsdD, E and F and/or the Hts system (Mazmanian *et al.*, 2003). The Hts system consists of a lipoprotein (HtsA) and two permeases (HtsB, HtsC) (Mason & Skaar, 2009), and also contributes to the uptake of iron-siderophore complexes (Beasley *et al.*, 2009). Inside the cell, either IsdG or IsdI degrades the porphyrin ring and releases free iron, or the haem complex is bound to membrane-associated proteins to act as a cofactor in, e.g. electron transport (Thöny-Meyer, 1997).

However, as high intracellular concentrations of haem damage DNA and proteins due to mechanisms of an oxidative (Everse & Hsia, 1997) and/or non-oxygen-dependent nature (our own unpublished observations), *S. aureus* needs to tightly regulate the release and uptake of this iron-containing molecule. Consequently, *S. aureus* is equipped with a system, termed HrtAB (haem-regulated transport system) (Reniere *et al.*, 2007), that is activated in the presence of increasing intracellular concentrations of haem. However, as *in vitro* studies have shown that increased concentrations of haemin (>10 µM) in the extracellular milieu suppress growth of this organism (Torres *et al.*, 2007), it appears that the HrtAB export mechanism is not efficient enough to protect *S. aureus* fully from elevated concentrations of haem. This suggests that *S. aureus* would benefit from a regulatory mechanism to modulate haemolysin expression and reduce lysis of erythrocytes, and thus decrease the release of haemoglobin at the infection site(s).

Recently, we have shown that in *S. aureus* the iron status is sensed via Fur, and that Fur mediates the abundance of a large number of exoproteins secreted by the model strain Newman and a number of clinical laboratory *S. aureus* isolates. Moreover, it could be demonstrated that in Newman the haemolytic and cytotoxic activity is also affected by *fur* (Torres *et al.*, 2010). Newman harbours a unique point mutation (Leu18→Pro18) in the sensor histidine kinase *saeS* that leads to a permanent activation of the sensor molecule of this two-component system (Geiger *et al.*, 2008; Schäfer *et al.*, 2009), yet this results in constitutive overexpression of class I *sae* target genes (*coa*, *fnbA*, *eap*, *sib*, *efb*, *fib*, *sae*) but not class II *sae* target genes, including the haemolysins *hla* and *hly* (Mainiero *et al.*, 2010). In addition to Fur, other regulators known to affect haemolysin expression and thus candidates for regulating red blood cell lysis are the Sae two-component regulatory system (a major regulator of haemolysin expression in *S.*

aureus) (Giraud *et al.*, 1997; Goerke *et al.*, 2005; Rogasch *et al.*, 2006; Yamazaki *et al.*, 2006), and the two-component system ArlRS, the SarA regulator, the *agr* system and the global stress component σ^B (reviewed by Bronner *et al.*, 2004).

Thus, to characterize a putative regulatory network connecting haemin/iron availability with haemolysin expression/haemolytic function as well as the underlying regulators, a detailed analysis employing various media (iron-rich, iron-poor), media supplements ($\pm$ haemin, iron sulphate or iron chelator) and *S. aureus* genetic backgrounds is needed. Using these tools, we show here that a functional *S. aureus* Sae system is a key switch regulating the production of haemolysins, suggesting that *sae* plays a major role in the protection of the microbial cell from exposure to cytotoxic concentrations of intracellular haem.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C and 150 r.p.m. in Luria-Bertani (LB) medium (Becton Dickinson) and RPMI 1640 (Invitrogen) supplemented with 1 % Casamino acids. Where required, media were supplemented with (per millilitre) 100 µg ampicillin, 7 µg chloramphenicol, 50 µg kanamycin or 10 µg tetracycline. The antibiotic resistance cassette-tagged *hla*, *saePQRS* and *fur* deletions of DU1090 (O'Reilly *et al.*, 1986) and Newman 29 (Geiger *et al.*, 2008) and MJH010 (Horsburgh *et al.*, 2001) were transduced into *S. aureus* LS1 using phage 85. Haemin MICs were determined by broth microdilution as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009).

Construction of the reporter gene plasmids pSB2035/hlap, pSB2035/hlbp and pSB2035/hlgAp. DNA fragments covering the *hla*, *hly* and *hlgA* promoters were amplified by PCR using primer pairs *hlap* forward/reverse (for pSB2035/hlap), *hlbp* forward/reverse (for pSB2035/hlbp) and *hlgAp* forward/reverse (for pSB2035/hlgAp), respectively, and LS1 DNA as template (primer sequences are listed in Table S1 available with the online version of this paper). The resulting PCR products were *EcoRI*/*SmaI*-digested and cloned into pSB2035 (Qazi *et al.*, 2001), thereby replacing the *agr* P3 promoter of pSB2035. Constructs harbouring the desired inserts were sequenced and used to electroporate RN4220, which served as donor for transducing the plasmids into LS1.

Quantitative real-time RT-PCR (qRT-PCR). Bacteria were grown in LB for 6 h, and the cultures were split into equal parts and supplemented with different concentrations of haemin, 2,2'-bipyridine and FeSO₄. The cultures were incubated for an additional 1 h at 37 °C and 150 r.p.m., and samples were removed at 15, 30 and 60 min after supplementation. RNA isolations and qRT-PCRs were carried out as described by Chatterjee *et al.* (2005). Levels of mRNA expression of the different genes were normalized against expression of the internal control *gyrB*. The amounts of different transcripts were expressed as the *n*-fold difference relative to the control gene ($2^{-\Delta C_T}$, where ΔC_T represents the difference in threshold cycle between the target and control genes).

Western blot analyses. Bacteria were grown in LB supplemented as indicated, and samples were taken after 6 h of growth. To account for differences in growth, samples were diluted with fresh LB to a OD₆₀₀ 1, and equal volumes were centrifuged at 5000 g for 10 min at 4 °C.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and phenotype*	Source or reference
<i>E. coli</i> strain		
DH5 α	F ⁻ ϕ 80d/ <i>lacZ</i> Δ M15 <i>recA1</i>	Invitrogen
<i>S. aureus</i> strains		
8325-4	NCTC8325 derivative lacking the prophages, <i>rsbU</i>	Novick (1967)
DU1090	8325-4 <i>hla-erm1</i> , pTS01, Erm ^R , Tet ^R	O'Reilly <i>et al.</i> (1986)
COL	MRSA, highly meticillin-resistant clinical isolate, Tet ^R	Dyke <i>et al.</i> (1966)
Cowan	ATCC12598, highly invasive clinical isolate	
LS1	Murine arthritis isolate, <i>saeS</i> ^{Leu} allele, highly haemolytic on SBA	Bremell <i>et al.</i> (1992)
LS1 Δ <i>fur</i>	LS1 Δ <i>fur</i> , Tet ^R , obtained by phage transduction from MJH010	This study
LS1 Δ <i>hla</i>	LS1 Δ <i>hla</i> , Erm ^R , obtained by phage transduction from DU1090	This study
LS1 Δ <i>sae</i>	LS1 Δ <i>sae</i> , Kan ^R , obtained by phage transduction from New 29	This study
MJH010	8325-4 Δ <i>fur</i> , Tet ^R	Horsburgh <i>et al.</i> (2001)
MSSA 1112	Clinical isolate, <i>bla</i> ⁺	Entenza <i>et al.</i> (1997)
N315	MRSA, clinical isolate	Kuroda <i>et al.</i> (2001)
Newman	ATCC25904, clinical isolate, ClfA/B and Eap hyperproducer, <i>saeS</i> ^{Pro} allele	Duthie (1952)
New 29	Newman Δ <i>saePQRS</i> , Kan ^R	Geiger <i>et al.</i> (2008)
New HG	Newman derivative with <i>saeS</i> ^{Leu} allele	Mainiero <i>et al.</i> (2010)
Plasmids		
pSB2035	<i>E. coli</i> – <i>S. aureus</i> shuttle plasmid, harbouring the <i>cat</i> gene conferring chloramphenicol resistance (Cm ^R), and a <i>gfp</i> – <i>lux</i> dual reporter plasmid harbouring the <i>agr</i> P3 promoter	Qazi <i>et al.</i> (2001)
pSB2035/ <i>hlap</i>	pSB2035 derivative, replacing the <i>agr</i> P3 promoter with an <i>hla</i> promoter fragment	This study
pSB2035/ <i>hlbp</i>	pSB2035 derivative, replacing the <i>agr</i> P3 promoter with an <i>hly</i> promoter fragment	This study
pSB2035/ <i>hlgAp</i>	pSB2035 derivative, replacing the <i>agr</i> P3 promoter with an <i>hlgA</i> promoter fragment	This study

*Cm^R, chloramphenicol resistant; Erm^R, erythromycin resistant; Kan^R, kanamycin resistant; Tet^R, tetracycline resistant. MRSA, meticillin-resistant *Staphylococcus aureus*.

The supernatants were used for Hla determinations, and cell pellets were used to isolate Eap by the lithium chloride extraction method described by Sobke *et al.* (2006). Protein samples were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and subjected to Western blot analyses with anti-Hla antibodies (Abcam) and anti-Eap antibodies (provided by J. Patti, Inhibitex), respectively.

Haemolytic activity assays. Haemolytic activities for LS1 and its derivatives were assayed on LB agar plates supplemented with 5% defibrinated sheep blood (SBA), human blood (HBA) and rabbit blood (RBA) after 24 h of growth at 37 °C followed by incubation at 4 °C for 24 h.

For determination of the haemolytic potential of *S. aureus* culture supernatants, overnight cultures were centrifuged at 5000 g for 10 min at 4 °C and the pellets resuspended in the same amount of fresh pre-heated medium. Cultures were then split into equal parts, and supplements added as indicated. Cultures were incubated for 2 h at 37 °C and 150 r.p.m., diluted with fresh LB to OD₆₀₀ 1, and centrifuged as described above. The supernatants were filter-sterilized and titrated for haemolytic activities as described by Nilsson *et al.* (1999). The highest dilution giving rise to visible lysis of erythrocytes was defined as the haemolytic titre.

Luciferase assay. For assaying luciferase activities of *S. aureus* strains harbouring the pSB2035 derivatives, cells were grown in LB supplemented with chloramphenicol. After 6 h of growth, cultures were split into two, and one half was supplemented with haemin to a final concentration of 4 μ M. Cultures were incubated for an additional 1 h. Then, 200 μ l samples of the cell suspensions were removed at the time points indicated and transferred to wells of a

96-well clear-bottomed black plate (Greiner). The plate was placed in a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer), and luminescence readings were taken for 5 s at 37 °C.

Statistical analyses. The statistical significance of changes between supplemented and unsupplemented cultures, and wild-type and mutant strains was assessed with the Wilcoxon signed-rank test (for related samples), and the Mann–Whitney U test (for independent samples), respectively. *P* values <0.05 were considered significant.

RESULTS AND DISCUSSION

Haemin affects growth of *S. aureus* in LB

Haemin is toxic for *S. aureus* if the concentration exceeds a certain value (Torres *et al.*, 2006). To assess the cytotoxic impact of haemin on growth of the highly haemolytic *S. aureus* strain LS1 (Bremell *et al.*, 1992), we determined its MIC and assayed growth in LB supplemented with different concentrations of haemin. Whereas LS1 was found to be relatively insensitive to haemin in the classical MIC assay (64 μ M), its growth was clearly affected by much lower concentrations of this iron-containing molecule if LS1 was cultured as a batch culture in LB at 150 r.p.m. and 37 °C (Fig. 1a). Concentrations of haemin as low as 2 μ M delayed the onset of growth of this isolate, and concentrations equal to or higher than 4 μ M were sufficient to significantly delay the growth of LS1 under these

Fig. 1. Impact of haemin on growth and haemolytic activity of *S. aureus*. (a) Growth characteristics of strain LS1 cultivated in LB supplemented with different concentrations of haemin. Data shown are the means $\pm$ SD of four independent experiments done in duplicate. Arrows indicate the time points of sampling for the determination of Hla contents and haemolytic activities in supernatants. (b) Haemolytic activities of supernatants of LS1 cultures grown for 6 h in the absence and presence of haemin (4 μ M) and 2,2'-bipyridine (250 μ M). * P <0.05, for unsupplemented versus supplemented cultures (Wilcoxon signed-rank test). (c) Hla secretion in response to haemin (4 μ M) and 2,2'-bipyridine (250 μ M). Cultures were grown to the time points indicated, and filter-sterilized supernatants were subjected to Western blot analysis using anti-Hla antibodies. The results are representative of at least two independent experiments.

conditions. Consequently, after 12 h of growth, the growth yields of the cultures supplemented with 4 and 8 μ M haemin, respectively, were significantly reduced compared with the unsupplemented control (mean $\pm$ SD OD₆₀₀ values of 1.92 ± 0.31 and 0.26 ± 0.21 for the cultures challenged with 4 and 8 μ M haemin, respectively, vs 3.56 ± 0.43 for the untreated control; P <0.001 between treated and untreated samples). However, haemin concentrations up to 4 μ M did not markedly affect the doubling times of the LS1 cultures during the exponential growth phase, which was significantly increased only in the LS1 culture challenged with 8 μ M haemin (89.52 ± 4.47 min for the haemin-supplemented culture vs 37.28 ± 1.14 min for the control; P <0.01). Moreover, after 24 h of growth, all cultures showed comparable OD₆₀₀ values, irrespective of the haemin addition, signalling that the haemin concentrations used here were not bacteriocidal for this strain (Table S2).

Haemin and 2,2'-bipyridine supplementation modulate α -haemolysin production and haemolytic activity of *S. aureus* LS1 in opposite directions

To study whether haemin might affect haemolysin production and its secretion in LS1, we tested the haemolytic activities and α -haemolysin (Hla) contents of supernatants of LS1 cells that were grown for 6 h either in the presence or in the absence of 4 μ M haemin. To account for differences in growth yields, cultures were adjusted with fresh LB to OD₆₀₀ 1 prior to sampling the supernatants. The haemin-supplemented supernatants of LS1 cultures showed a significantly reduced capacity to lyse human erythrocytes, as compared with the control (Fig. 1b). Similarly, we detected a strong reduction in Hla in the haemin-supplemented culture supernatants after 6 h of growth (Fig. 1c). To rule out that growth phase-dependent differences between the haemin-supplemented culture and the untreated control (Fig. 1a) might account for this effect, we determined the Hla contents in the supernatants of early stationary phase cultures (OD₆₀₀ 3). We again found a decrease in Hla in the haemin-supplemented culture supernatants, although this was less pronounced than that observed after 6 h of growth (Fig. 1c). As we have recently observed that iron depletion increases the production of Hla and the haemolytic activity of *S. aureus* strain Newman (Torres *et al.*, 2010), we also tested here whether and how the addition of the iron scavenger 2,2'-bipyridine (250 μ M) affected the haemolytic activity and Hla expression of strain LS1. In line with our previous findings, we observed a significant increase in haemolytic activity in the supernatants of the 2,2'-bipyridine-challenged cultures (Fig. 1b), and detected elevated Hla signals in these culture supernatants (Fig. 1c). These data strongly suggest that subinhibitory amounts of haemin reduce the haemolytic activity and α -haemolysin production and/or secretion of *S. aureus*, while iron-limiting conditions trigger the opposite effects by upregulating Hla production/secretion and increasing the haemolytic activity.

Subinhibitory concentrations of haemin affect the transcription of haemolysins in *S. aureus*

To determine how haemin affects the haemolysin production of *S. aureus*, we next analysed the transcription of four major haemolysins of this pathogen, *hla* (encoding α -haemolysin), *hly* (encoding β -haemolysin), *hlgA* (encoding the S-class component of γ -haemolysin) and *hld*, which is encoded by RNAIII of the *agr* locus, in response to haemin. To eliminate potential growth phase-dependent differences in haemolysin transcription in this assay, LS1 cells were grown for 6 h in LB and split into two parts, and one part was supplemented with haemin (4 μ M). Samples for qRT-PCR were taken immediately after addition of haemin (time point 0), and 15, 30 and 60 min later (Fig. 2a, c). Addition of subinhibitory concentrations of haemin resulted in significant reductions in transcription for *hly* and *hlgA* as early as 15 min after the supplementation. A similar, but not significant, trend was observed for *hla* transcription. No such difference was observed for RNAIII transcription between the unsupplemented and the supplemented samples (data not shown).

The repressive effect of haemin on the transcription of haemolysins appears to be independent of growth media

To investigate the impact of the growth medium on the effect of haemin on haemolysin expression, we reproduced the experiment in a chemically defined medium, RPMI 1640 (hereafter RPMI), which contained smaller amounts of iron (approx. 0.5 μ M) (Fenwick *et al.*, 1996) than LB (approx. 20 μ M) (Rayner & Sadler, 1990). In RPMI, strain LS1 produced nearly 60 times less *hla* transcripts than in LB after 6 h of growth, confirming recent findings that *hla* transcription is highly media-dependent (Ray *et al.*, 2009). Transcription of *hly* was also found to be affected by the growth medium, and was 120-fold increased in RPMI, while *hlgA* transcription varied only by a factor of 3 between LB and RPMI (Table 2). Addition of haemin to LS1 cells grown in RPMI for 6 h, however, again resulted in highly significant decreases in *hly* and *hlgA* transcript levels when compared with the unsupplemented control (Fig. 2a). Transcription of *hla*, although strongly influenced by the growth medium in its transcription, was not affected by the addition of haemin.

We confirmed the haemin effect on haemolysin transcription with promoter–reporter gene fusion experiments. We created a series of promoter–reporter gene fusion constructs based on *agrP3-gfp-lux* plasmid pSB2035 (Qazi *et al.*, 2001), in which the *agr* P3 promoter was replaced by the *hla*, *hly* and *hlgA* promoters. The plasmids were used to transform strain LS1, and the impact of haemin supplementation (4 μ M) on the activities of the haemolysin promoters was determined as changes in luciferase activities. In line with our qRT-PCR data, we observed the strongest luciferase activity values with the LS1 derivative harbouring the *hla* promoter construct [773 ± 224 relative light units (RLU)

after 6 h of growth in LB]. Luciferase activity values with the LS1 derivative harbouring the *hlgA* promoter construct were more than 100-fold smaller than those found for the *hla* construct (6.4 ± 1.6 RLU after 6 h of growth). However, we failed to detect luciferase activity with the LS1 derivative harbouring the *hly* promoter construct, which was indistinguishable from the background luminescence of our test system. Addition of haemin to the 6 h LB cell cultures of LS1 derivatives harbouring the *hla* and *hlgA* promoter–reporter gene fusion constructs, resulted in a threefold reduction in luciferase activities for the strain harbouring the *hlgA* promoter construct, while luciferase activities were not affected by haemin in LS1 transformed with the *hla* promoter–reporter gene fusion construct (Fig. 2b). Although in line with our qRT-PCR data, the *hla* promoter–reporter gene fusion results are at first sight in conflict with our Western blot data demonstrating decreased Hla contents in haemin-supplemented culture supernatants (Fig. 1c). However, as Hla production and its stability are markedly affected by post-transcriptional and post-translational mechanisms (Morfeldt *et al.*, 1995; Lindsay & Foster, 1999), we cannot exclude here that the observed decrease in Hla in the supernatants of haemolysin-challenged cultures might be due to haemin-induced alterations in *hla* translation or protease activities.

Iron sulphate affects haemolysin transcription in a media-dependent fashion

To test whether the observed effect on haemolysin transcription is haemin-dependent or could be induced by other iron sources as well, LS1 cells were grown for 6 h in either LB or RPMI. Subsequently, increasing amounts of haemin (1–4 μ M) or FeSO₄ (1–4 μ M) were added to the cultures (Fig. 3). In the iron-rich medium LB, addition of FeSO₄ did not significantly alter the transcription of *hla*, *hly* or *hlgA* at any of the concentrations tested. Supplementation of LB with increasing amounts of haemin, on the other hand, decreased the transcription of *hly* and *hlgA*. None of the conditions tested altered the transcription of *hla* significantly, although higher concentrations of FeSO₄ seemed to foster transcription. In the iron-poor medium RPMI, the addition of either FeSO₄ or haemin resulted in clear reductions in *hly* and *hlgA* transcription (Fig. 3a), and FeSO₄ seemed to repress the transcription of these two genes even more strongly than the equimolar amounts of haemin. However, as in LB, neither of the concentrations of haemin tested significantly altered the transcription of *hla* in RPMI. Interestingly, the haemin-induced repressive effect on the transcription of *hly* and *hlgA* was still effective under growth conditions in which *S. aureus* suffered from iron-limitation, as addition of FeSO₄ clearly increased the growth of the LS1 culture in RPMI (data not shown). This suggests that the repressive effect of haemin is independent of the iron content of the growth medium. The haemolysin gene transcription-regulating mechanisms that were effective under iron-limiting conditions, on the other hand, appeared to be

Fig. 2. Transcription of haemolysin genes and *eap* in LS1 in response to haemin. (a) Effect of haemin supplementation (4 μ M) on the transcription of haemolysin genes in LS1 cells grown in LB and RPMI. Cells were grown for 6 h before haemin was added, and samples were taken at the time points indicated. Changes in transcription were assayed by qRT-PCR. Data shown are the means $\pm$ SD of three independent experiments performed in duplicate. (b) Impact of haemin supplementation (4 μ M) on luciferase activities of cultures of LS1 derivatives harbouring plasmids pSB2035/hlap and pSB2035/hlgAp. Cells were cultured in LB for 6 h before haemin (4 μ M) was added. Luciferase activities were determined at the time points indicated. Data shown are the means $\pm$ SD of seven independent experiments. (c) Effect of haemin supplementation (4 μ M) on transcription of *eap* in LS1 cells grown in LB. * P <0.05, ** P <0.01 for unsupplemented versus supplemented cultures (Wilcoxon signed-rank test). (d) Western blot analysis of Eap in cell-wall extracts of LS1 cells grown for 6 h in LB in the presence of different concentrations of haemin. The result is representative of at least two independent experiments.

insensitive to the iron source, as they were affected by both haemin and FeSO₄ supplementation.

Effect of haemin supplementation on the activity of selected global regulators

To identify regulatory molecules that might exert the haemin effect on haemolysin transcription, we tested the impact of haemin supplementation on the transcription of selected regulators known to affect haemolysin expression, such as *ArlRS* (Liang *et al.*, 2005), *SaePQRS* (Giraud *et al.*, 1997; Goerke *et al.*, 2001, 2005; Li & Cheung, 2008; Xiong *et al.*, 2006), *SarA* (Goerke *et al.*, 2001; Xiong *et al.*, 2006) and σ^B (Bischoff *et al.*, 2004; Cheung *et al.*, 1999; Horsburgh *et al.*, 2002; Goerke *et al.*, 2005). The addition of haemin to LS1 cells grown in LB did not affect the transcription of *arlRS*, *sarA* or *sigB* (data not shown). However, in LB a strong repression in *sae* transcription was observed after the addition of haemin (Fig. 3b), indicating that this iron-containing porphyrin molecule might affect the activity of the Sae two-component regulatory system. Moreover, similarly to *hla* and *hly*, *sae* expression was found to be greatly affected by the medium: when LS1 cells were grown in RPMI, *sae* transcription was found to be ~60-fold decreased when compared with LB (Table 2). Supplementing RPMI with haemin resulted in a slight (and significant only at 4 μ M) reduction in *sae* transcription, whereas FeSO₄ had a stronger effect (Fig. 3b). However, the expression patterns of *sae* upon haemin and FeSO₄

supplementation in both media were similar to those found for *hly* and *hlgA* (Fig. 3a).

The *sae* locus is required for the effect of haemin on the transcription of haemolysins

We and others have reported recently that the ferric uptake regulator Fur modulates haemolysin production and Sae activity in *S. aureus* strain Newman under iron-limiting conditions (Johnson *et al.*, 2011; Torres *et al.*, 2010). To test whether Sae and/or Fur are required for mediating the haemin effect on haemolysin transcription, we inactivated the *sae* and *fur* loci in LS1. We first determined the impact of the mutations on haemolytic activities after growth on SBA, RBA and HBA plates (Fig. 4a). While the wild-type showed clear haemolytic zones surrounding the colonies on all plate types tested, this was not the case with the mutants. Inactivation of the *sae* locus in LS1 resulted in a marked decrease of the haemolytic areas surrounding the mutant colonies, and this phenotype was observed on all blood agar plate types. The LS1 Δfur mutant, on the other hand, produced haemolytic zones on SBA and RBA plates that were comparable with those found with the wild-type. On agar plates supplemented with human blood, however, LS1 Δfur produced almost no haemolytic areas, and this phenotype was associated with a clearly decreased colony size of the mutant. Interestingly, an LS1 Δhla mutant, incapable of producing α -haemolysin, formed smaller haemolytic areas on RBA but not on SBA or HBA plates,

Table 2. Effect of growth medium on transcription after 6 h of growth

Strain	Growth medium	Transcripts (copy/copy of <i>gyrB</i>)*			
		<i>hla</i>	<i>hly</i>	<i>hlgA</i>	<i>sae</i>
LS1	LB	1631.9 $\pm$ 964.1	0.3 $\pm$ 0.1	1.3 $\pm$ 0.5	696.8 $\pm$ 371.4
	RPMI	26.3 $\pm$ 10.4	43.8 $\pm$ 20.2	0.4 $\pm$ 0.05	10.8 $\pm$ 2.8
LS1 Δsae	LB	0.003 $\pm$ 0.0006	0.02 $\pm$ 0.005	0.01 $\pm$ 0.002	—
	RPMI	0.07 $\pm$ 0.03	0.06 $\pm$ 0.02	0.01 $\pm$ 0.004	—
LS1 Δfur	LB	11.2 $\pm$ 5.1	0.6 $\pm$ 0.2	4.7 $\pm$ 3.1	3.2 $\pm$ 0.6
	RPMI	115.63 $\pm$ 99.75	66.3 $\pm$ 61.9	0.2 $\pm$ 0.1	4.8 $\pm$ 0.9

*Transcript levels were determined by the 2^{- ΔC_T} method. Data are means $\pm$ SD of at least three independent experiments performed in duplicate.

Fig. 3. Effect of haemin and FeSO_4 supplementation on transcription of haemolysin genes and of *saeS* in *S. aureus*. Cells were grown for 6 h in LB and RPMI, before the haemin or FeSO_4 concentrations indicated were added, and samples were taken 30 min after addition of the substances. (a) Effects on haemolysin gene transcription in LS1. (b) Effects on *saeS* transcription in LS1. (c) Effect of haemin supplementation (4 μM) on *hlgA* transcription in different *S. aureus* lineages. Changes in transcription were assayed by qRT-PCR. Data shown are the means $\pm$ SD of fold changes between supplemented and unsupplemented controls of three independent experiments done in duplicate. * P <0.05, ** P <0.01, *** P <0.001 for unsupplemented versus supplemented cultures (Wilcoxon signed-rank test).

Fig. 4. Effects of *fur*, *hla* and *sae* deletions on the haemolytic capacity of LS1. (a) Cells were grown for 24 h at 37 °C on agar plates supplemented with sheep blood (SBA), human blood (HBA) and rabbit blood (RBA), followed by 24 h incubation at 4 °C. (b) Haemolytic activities of supernatants of LS1 and its derivatives on human (HB) and rabbit erythrocytes (RB). (c) Effect of haemin supplementation (4 μ M) on transcription of *hla*, *hlb*, *hlgA* and *saeS* of strain LS1 and its derivatives grown in LB and RPMI. Cells were grown for 6 h before haemin was added, and samples were taken 30 min after addition of the substances. Changes in transcription were assayed by qRT-PCR. Data shown are the means $\pm$ SD of fold changes between supplemented and unsupplemented controls of three independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for unsupplemented versus supplemented cultures (Wilcoxon signed-rank test). WT, wild-type.

indicating that Hla, although the predominantly expressed haemolysin of this strain, was not essential for the haemolytic activity of LS1 on human red blood cells.

Second, we assayed the haemolytic potentials of supernatants of OD₆₀₀-adjusted overnight cultures of LS1 and its derivatives on human and rabbit erythrocytes (Fig. 4b). These analyses essentially confirmed our findings described above: whereas supernatants of LS1 Δsae produced no visible haemolytic activities in these assays, LS1 Δfur supernatants showed haemolytic activities that were comparable with those obtained with the wild-type supernatants. Interestingly, supernatants of LS1 Δhla showed haemolytic activities on human erythrocytes that were in the range of those found with the wild-type and LS1 Δfur supernatants, while haemolysis of rabbit erythrocytes was clearly decreased with the LS1 Δhla supernatants when compared with the wild-type and LS1 Δfur .

Third, we assayed the impact of the mutations on the transcription of the haemolysin genes. Inactivation of both *sae* and *fur* clearly affected the transcription of haemolysin genes, although this effect was highly media-dependent (Table 2). However, addition of subinhibitory concentrations of haemin to LS1 Δfur cells grown in LB and RPMI yielded similar reductions in *hlb*, *hlgA* and *saeS* transcript levels as in LS1 wild-type cells, while inactivation of *sae* in LS1 abolished the haemin-induced reduction of haemolysin transcription (Fig. 4c). Transcription of *hla* was apparently not affected by the addition of haemin to either of the mutant cultures.

These findings suggest that Hla is not a major player in the haemolysis phenotype observed with LS1, and indicate that Hla is not essential to the effect of haemin on haemolysis of human erythrocytes. Interestingly, although *hla* transcription was severely downregulated in *saeRS* deletion strains (Giraud *et al.*, 1997; Goerke *et al.*, 2001, 2005; Xiong *et al.*, 2006), and direct binding of the response regulator SaeR to the *hla* promoter has been recently demonstrated (Nygaard *et al.*, 2010; Sun *et al.*, 2010), *hla* transcription was not

significantly affected by haemin-mediated *sae* repression in LS1, while *hly*, *hlgA* and *saeS* responded to this stimulus in an Sae-dependent manner. It has been shown that *sae* target genes differ significantly regarding SaeRS dosage and SaeS-sensing mechanisms. For instance, *hla* expression is not affected by mutations in SaeS as present in strain Newman and is rather insensitive towards SaeRS dosage (Mainiero *et al.*, 2010). The data presented here suggest that the Sae-dependent modulation of haemolysin genes in *S. aureus* might be even more complicated.

S. aureus strain Newman differs in its haemin effect on *hlgA* transcription

To test whether the repressive effect of haemin on haemolysin transcription is restricted to *S. aureus* strain LS1, we analysed the impact of haemin supplementation on the transcription of *hlgA* in a number of laboratory strains (Fig. 3c). With the exception of wild-type strain Newman (harbouring a Pro18 mutation in *saeS*), all other isolates tested, including the Newman derivative New HG, which contains a repaired *saeS* gene (*saeS*^{Leu18}) (Mainiero *et al.*, 2010), showed the same effect as LS1, although the repression intensities differed from strain to strain.

In the context of our previous findings (Torres *et al.*, 2010), our new observations underline the importance of strain background and media when assessing the consequences of haemin, iron exposure or iron restriction on regulation and target gene expression in *S. aureus*. In our previous study, a *fur* mutant of strain Newman displayed not only elevated total exoprotein production but also increased haemolysis and *hla* expression upon cultivation in an iron-rich medium. In the *fur* mutant of strain LS1, we also observed increased *hla* transcription, but only in the low iron-containing medium RPMI. Cultivation of LS1 Δfur cells in the iron-rich medium LB, on the other hand, resulted in a strong decrease in *hla* transcription, contrary to our previous findings and those of Johnson *et al.* (2011) with strain Newman. An apparent explanation for this discrepancy might be the leucine to proline mutation at position 18 in SaeS of strain Newman, which leads to a constitutive activation of the Sae system in this genetic lineage (Adhikari & Novick, 2008; Mainiero *et al.*, 2010). We and others have shown that the Newman *sae* system responds differently to environmental stresses (Geiger *et al.*, 2008; Schäfer *et al.*, 2009) from a Newman derivative harbouring a leucine at position 18 of SaeS (SaeS^{Leu}), and that a Newman SaeS^{Pro} derivative expresses exoproteins differently from Newman wild-type (Adhikari & Novick, 2008; Luong *et al.*, 2011; Mainiero *et al.*, 2010). In line with this, we found that a Newman derivative harbouring an *saeS*^{Leu} allele, New HG, responded to haemin exposure similarly to strain LS1 and a number of other *S. aureus* genetic backgrounds, while strain Newman did not (Fig. 3c), strongly suggesting that the response of the Sae system to environmental signals is severely altered by the *saeS* mutation of strain Newman.

Subinhibitory concentrations of haemin and 2,2'-bipyridine affect Sae activity

Although Sae is known to affect its own expression (Adhikari & Novick, 2008), differences observed in *sae* transcription do not necessarily reflect Sae activity, as other regulators have been reported to modulate the transcription of this regulatory locus too (Giraud *et al.*, 2003; Li & Cheung, 2008; Novick & Jiang, 2003). To test whether haemin and 2,2'-bipyridine affect not only *sae* transcription but also Sae activity, we analysed the impact of haemin and 2,2'-bipyridine supplementation, on the expression of the extracellular adherence protein (Eap), which was previously shown to be dependent on Sae activity in its expression (Harraghy *et al.*, 2005). Similar to our observations made for *hly* and *hlgA*, addition of 4 μ M haemin to an LS1 culture grown for 6 h in LB resulted in an immediate reduction in

Fig. 5. Effect of 2,2'-bipyridine supplementation on the transcription of *saeS* and *hlgA*, and on Eap production in LS1. (a) LS1 cells were grown for 6 h in LB before 2,2'-bipyridine (250 μ M) was added, and samples were taken at the time points indicated. Changes in transcription were assayed by qRT-PCR. Data shown are the means $\pm$ SD of three independent experiments performed in duplicate. * P <0.05, ** P <0.01 for unsupplemented versus supplemented cultures (Wilcoxon signed-rank test). (b) Western blot analysis of Eap in cell-wall extracts of LS1 cells grown for 6 h in LB in the presence of the 2,2'-bipyridine concentrations indicated. The result is representative of at least two independent experiments.

Fig. 6. Proposed model for the regulation of *S. aureus* haemolytic activity in response to iron availability and haem. Iron limitation (1) leads in *S. aureus* to an increased production and secretion of haemolysins via Fur and SaeR/S (2), which disintegrate erythrocytes to release haemoglobin (3). The released iron-storage protein is bound by the bacterium to particular receptors, and the haem subunit is released and imported into the cytoplasm (4). The increased intracellular/extracellular haem concentration is sensed by the SaeR/S system to decrease SaeS activity, which leads to a downregulation of haemolysin production (5) to prevent intoxication of the bacterium by this molecule. The ongoing uptake/consumption of extracellular haemoglobin/haem by *S. aureus* (6) depletes the iron in the extracellular milieu, which restarts the cycle (1).

eap transcription, suggesting that Sae activity is altered by the presence of subinhibitory concentrations of this molecule (Fig. 2c). This hypothesis is further strengthened by findings obtained from Western blot analyses performed with cell-wall extracts of LS1 cells that were cultured in the absence and presence of increasing concentrations of haemin. Haemin concentrations as low as 1 μ M were sufficient to reduce the amount of this cell wall-associated adhesin, while 4 μ M haemin nearly abolished the production of Eap (Fig. 2d). Addition of the iron scavenger 2,2'-bipyridine (250 μ M) to a 6 h LB culture of LS1, on the other hand, yielded an immediate and significant increase in *sae* and *hlgA* transcription (Fig. 5a). Similarly, growth of LS1 in LB supplemented with 2,2'-bipyridine increased Eap quantity on the bacterial cell surface markedly (Fig. 5b), in line with recent observations showing that low iron conditions affect Sae activity in a Fur-dependent manner (Johnson *et al.*, 2011).

Conclusions

Haemoglobin is considered to be the major iron source for *S. aureus* during infection (Lee *et al.*, 2008). Accordingly, the pathogen is equipped with a number of factors, allowing it (i) to release haemoglobin from red blood cells,

(ii) to bind haemoglobin and to import the iron-containing porphyrin (haem), (iii) to degrade the imported haem and to release the iron, and (iv) to protect itself from the cytotoxic effects that are associated with this molecule. Here, we show that the *sae* system is likely to contribute to this complex regulatory circuit by modulating the haemolysin production of *S. aureus* in response to iron limitation and haem availability. Based on our transcriptional data, we propose that *S. aureus* regulates its haemolytic potential in an Sae- and Fur-dependent manner (Fig. 6). Under iron-limiting conditions, *S. aureus* activates the production and secretion of haemolysins via the Sae and Fur regulatory systems, while higher (subinhibitory) concentrations of haemin in the extracellular environment of *S. aureus* – probably signalling to the bacterium that iron is available in sufficient amounts – lead to a downregulation of haemolysin production that is due to a repression of Sae activity. However, the molecular mechanism(s) by which haemin can interact with the Sae regulatory system to modulate its activity still need to be identified.

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