Arch Microbiol (2011) 193:23–34
DOI 10.1007/s00203-010-0641-1

ORIGINAL PAPER

Searching for small σ^B-regulated genes in *Staphylococcus aureus*

Jesper S. Nielsen · Mie H. G. Christiansen · Mette Bonde · Sanne Gottschalk · Dorte Frees · Line E. Thomsen · Birgitte H. Kallipolitis

Received: 3 August 2010 / Revised: 25 August 2010 / Accepted: 9 September 2010 / Published online: 27 October 2010

Abstract In recent years, small RNAs (sRNAs) have been identified as important regulators of gene expression in bacteria. Most sRNAs are encoded from intergenic regions and are only expressed under highly specific growth conditions. In *Staphylococcus aureus*, the alternative sigma factor, σ^B, is known to contribute to the overall stress response, antibiotic resistance, and virulence. The σ^B regulon in *S. aureus* is well described and comprises approximately 200 annotated genes, including several genes encoding virulence factors. In the present study, we have identified three novel σ^B-dependent transcripts encoded from genomic regions previously annotated as intergenic. All three transcripts, named SbrA, SbrB, and SbrC, are highly conserved in *S. aureus,* and we confirmed their presence in four different isolates (SH1000, Newman, COL, and UAMS-1). Curiously, two of these genes (*sbrA* and *sbrB*) were found to contain open reading frames encoding small, highly basic peptides that are conserved among *Staphylococci*. The third transcript (SbrC) did not contain any likely open reading frame and thus constitute a genuine non-coding sRNA. The functions of these genes are currently unknown but are likely to be important for the σ^B-mediated response of *S. aureus* to adverse conditions.

Keywords *S. aureus* · sRNA · σ^B

Communicated by John Helmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-010-0641-1) contains supplementary material, which is available to authorized users.

J. S. Nielsen · M. H. G. Christiansen · M. Bonde · B. H. Kallipolitis (✉)
Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
e-mail: bhk@bmb.sdu.dk

S. Gottschalk · D. Frees · L. E. Thomsen
Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C, Denmark

Introduction

The gram-positive human pathogen *Staphylococcus aureus* is the causative agent of a wide range of nosocomial and community-acquired infections. The pathogenesis of *S. aureus* is highly complex and relies on a variety of virulence-associated factors, including adhesins, toxins, exoenzymes, and immune-modulating factors (Lowy 1998). The production of virulence factors at the appropriate time and place during infection is controlled by the coordinated actions of multiple transcriptional regulatory proteins, the alternative sigma factor σ^B, and the regulatory RNAIII (Novick 2003).

Staphylococcus aureus encodes four sigma factors: the housekeeping σ^A, the stress responsive σ^B (Wu et al. 1996), σ^H, which may be involved in natural competence (Morikawa et al. 2003), and σ^S, which appears to be a component of the stress and virulence responses (Shaw et al. 2008). The best studied of the three alternative sigma factors is σ^B, which is involved in stress responses and antibiotic resistance, and contributes to pathogenesis in some, but not all, infection models tested so far (Jonsson et al. 2004;Giachino et al. 2001;Horsburgh et al. 2002a; Kullik et al. 1998; Kullik and Giachino 1997; Lorenz et al. 2008). Proteomics and microarray studies have shown that the σ^B regulon consists of around 200 genes, some of which are involved in virulence, membrane transport processes, and cell wall metabolism

(Bischoff et al. 2004; Gertz et al. 2000; Pane-Farre et al. 2006). Curiously, multiple σ^B-regulated genes do not contain a consensus σ^B promoter, and several genes are under negative control by σ^B suggesting that these genes are affected by σ^B in an indirect manner, most likely through the actions of σ^B-regulated gene regulatory factors.

RNAIII is the major effector molecule of the quorum-sensing system *agr* which controls the expression of numerous virulence genes in *S. aureus* (Novick 2003). RNAIII belongs to a relatively new group of bacterial gene regulators, the *trans*-encoded base pairing small RNAs (sRNAs), which control gene expression by forming imperfect duplexes with specific target mRNAs, thereby affecting their translation and/or stability (Waters and Storz 2009). RNAIII acts as an antisense RNA to stimulate translation of the *hla* mRNA (encoding α-hemolysin) and to repress translation of several mRNAs, including the *spa* and *rot* mRNAs, encoding protein A and the virulence regulator Rot (repressor of toxins), respectively (Geisinger et al. 2006; Boisset et al. 2007). In gram-negative species, most base pairing sRNAs depend on the RNA chaperone Hfq, which serves to both stabilize the sRNAs and facilitate their interaction to target mRNA (Aiba 2007). In contrast to this, Hfq has been shown to be dispensable for RNAIII-dependent riboregulation in *S. aureus* (Boisset et al. 2007; Geisinger et al. 2006; Bohn et al. 2007). Intriguingly, whereas most base pairing sRNAs studied so far appear to be non-coding, RNAIII has been found to encode a small protein, the 26-amino acid δ-hemolysin. Thus, RNAIII belongs to the category of dual-function sRNA regulators, which also includes the *Eschericia coli* SgrS RNA (Wadler and Vanderpool 2007) and the SR1 RNA in *Bacillus subtilis* (Gimpel et al. 2010).

Over the past decade, numerous sRNAs have been identified in bacteria using a variety of computational and experimental approaches (Gertz et al. 2000; Sharma and Vogel 2009). In *S. aureus*, computational searches in combination with expression studies have revealed the presence of multiple sRNA encoded from the intergenic regions (IGRs) (Geissmann et al. 2009; Pichon and Felden 2005; Bohn et al. 2010). Interestingly, seven sRNAs were found to be encoded from pathogenicity islands suggesting a role in virulence (Pichon and Felden 2005). Furthermore, the expression of four non-coding sRNAs was recently shown to be controlled by σ^B or *agr* (Geissmann et al. 2009). Thus, the gene regulatory circuits controlling virulence gene expression in *S. aureus* are likely to include multiple regulatory sRNAs besides RNAIII. In addition to sRNA-encoding genes, the IGRs in bacteria most likely contain multiple unannotated genes encoding small proteins (i.e., proteins < 50 amino acids), which have been overlooked so far due to their small size. In line with this, recent systematic investigations of the IGRs in *E. coli* resulted in the identification of more than 60 genes encoding small proteins, many of which appear to be involved in the general stress response (Hemm et al. 2010; Hobbs et al. 2010; Hemm et al. 2008).

In the present study, we searched for genes encoding σ^B-dependent sRNAs in *S. aureus*. Using a bioinformatics approach, we predicted the existence of five small σ^B-regulated genes within the IGRs of *S. aureus* strain N315. Three small σ^B-dependent transcripts were verified experimentally in *S. aureus* strains SH1000, COL, Newman, and UAMS-1. None of the transcripts required Hfq for expression or stability. Two genes, named *sbrA* and *sbrB* (for Sigma*B*-dependent small *R*NA *A* and *B*), were found to contain small open reading frames (ORFs) encoding putative small proteins of 26 and 38 amino acids, respectively. Curiously, the ORF within *sbrB* appears to be translated in some, but not all of the *S. aureus* strains tested. The third transcript (SbrC) is likely to be a genuine non-coding sRNA. In conclusion, this study adds three novel genes to the σ^B regulon in *S. aureus*. Apparently, these genes have been overlooked in previous studies on σ^B-dependent genes in *S. aureus*, because of their non-coding nature and/or small size.

Results and discussion

Prediction of small σ^B-regulated genes in the IGRs of *S. aureus*

Genes encoding sRNAs can be predicted bioinformatically by a variety of methods. When reliable consensus sequences are available for alternative sigma factors and/or transcription factors, they provide an attractive starting point because they act as filters against false positives. Indeed, such an approach was successful in identifying novel σ^B-regulated sRNAs in *Listeria monocytogenes* (Nielsen et al. 2008). Here, we have used a similar approach to predict the existence of putative σ^B-regulated sRNAs within the IGRs of *S. aureus* strain N315. A detailed overview of our search can be found in the "Experimental procedures" section. Briefly, using the AureoList webpage (AureoList 2010), we searched for the presence of σ^B consensus sites [GWWT_$N_{14–17}$_GGGWWW (Pane-Farre et al. 2006)] within the IGRs of *S. aureus* strain N315. Putative candidates were identified by the presence of a downstream intrinsic terminator. This approach resulted in the prediction of five putative σ^B-regulated genes, none of which contain annotated ORFs in *S. aureus* N315. An overview of these is shown in Table 1.

Experimental validation of σ^B-dependent transcripts

In order to validate the presence of an actual transcript from the predicted loci, we performed northern blotting

Table 1 Putative σ^B-regulated genes within IGRs of *S. aureus* strain N315

Name	Flanking genes	σ^B consensus	Length	Intrinsic terminator
sbrA	SA0891 > < < SA0892	GTTT-N_{17}-GGGTAT	~200	AATACAGCGCCACTCTTATATGATTTACGATAGCGCTGTATTTTT
sbrB	SA1173 > > < SA1174	GTTT-N_{16}-GGGTAA	~150	GGCTCGCTCCTGTAAATTATTACGGGGGCGAGTTTT
sbrC	SA0586 < > < SA0587	GTAT-N_{14}-GGGTAA	~310	AACACTTGTTTCTACTAATAAGTGTTTT
sbrD	SA0040 > < > SA0041	GTAT-N_{14}-GGGTAT	~125	CAGAGGAAATATTCAACGACTTGATTGTTTCCTCTGTTTT
sbrE	SA1519 > > < SA1520	GAAT-N_{17}-GGGTTT	~290	CGCCTTTCCATTGTTGATAAATGGAAAGACGTTTTTTT

on cells subjected to KOH stress, which is known to be a potent σ^B activating agent (Pane-Farre et al. 2006). For the initial experimental validation of *sbrA*, *sbrB*, *sbrC,* and *sbrE,* we used *S. aureus* strain SH1000, whereas in the case of *sbrD*, the MRSA strain ATCC 33591 was employed. The results of the northern blot experiments are shown in Fig. 1. In the case of *sbrA*, *sbrB,* and *sbrC*, the exposure of SH1000 wild-type cells to KOH resulted in the induction of small transcripts of approximately 200 nucleotides (nt), 150 and 300 nt, respectively (Fig. 1a–c). Importantly, none of the transcripts were observed in a SH1000Δ*sigB* mutant strain. Thus, these experiments verify the existence of SbrA, SbrB, and SbrC RNAs in SH1000 and show that their expression is completely dependent on σ^B. In contrast to this, no KOH-inducible transcript corresponding to SbrD or SbrE were detected in MRSA strain ATCC 33591 or SH1000, respectively, and we therefore do not consider *sbrD* and *sbrE* any further in the present study (data not shown). The genomic localization of *sbrA-C* and MFOLD predicted secondary structures of SbrA, SbrB and SbrC are shown in Fig. 1a–c.

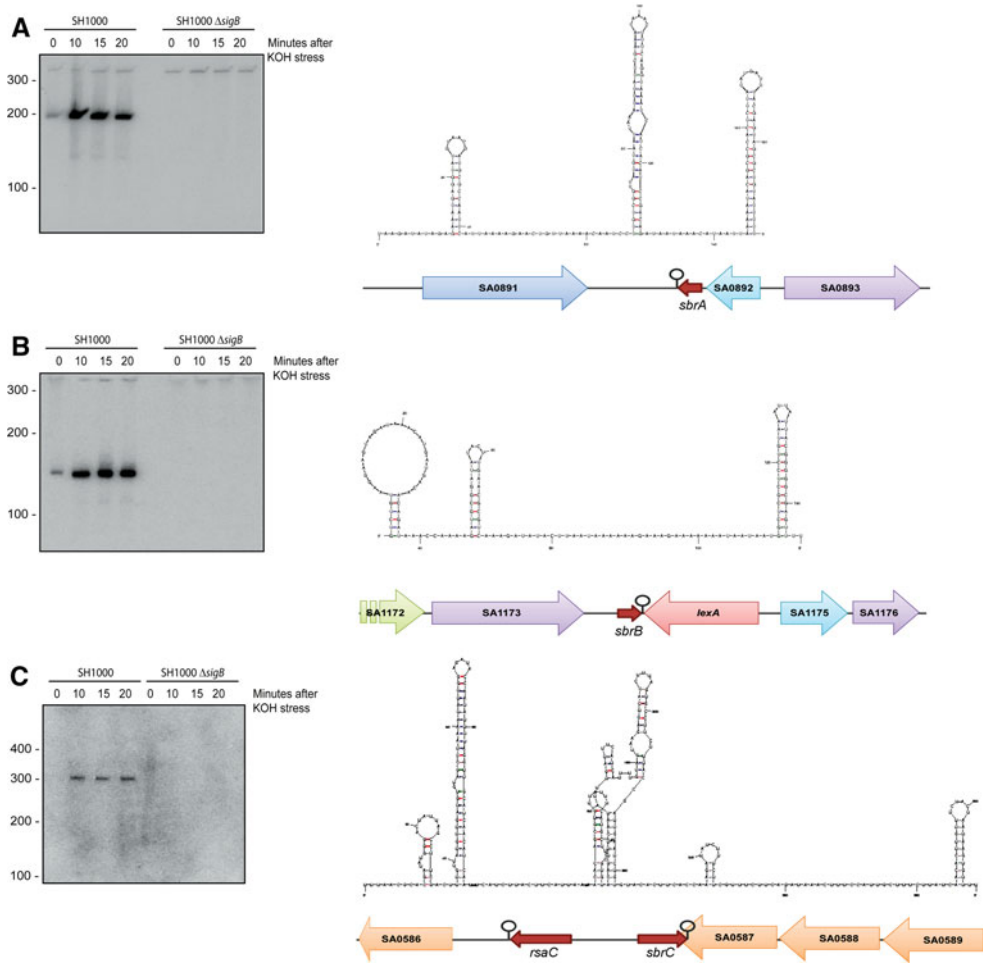

Fig. 1 Identification of putative σ^B-regulated sRNAs by northern blotting. For the identification of *sbrA* RNA (**a**), *sbrB* RNA (**b**), and *sbrC* RNA (**c**), *S. aureus* strain SH1000 and its isogenic Δ*sigB* strain were employed. For northern blot experiments (*left*), cells were grown to early exponential phase at which point σ^B activity was induced for 20 min by the addition of KOH to a final concentration of 30 mM. RNA was extracted as described in the "Experimental procedures" section at the indicated time points following stress induction. The predicted secondary structures of *sbrA*, *sbrB,* and *sbrC* RNAs (*right*) was determined by MFOLD (MFOLDi 2010). The schematic representation of the genomic context is not drawn to scale

Mapping the transcription start sites of the *SbrA* and *SbrB* RNAs

The *sbrA* gene is situated in the IGR between SA0892 and SA0891 (both encoding unknown proteins) and appears to share its intrinsic terminator with its upstream gene, SA0892 (Fig. 1a). The expected length of the SbrA RNA is 197 nt, which is in agreement with the size of the transcript observed by northern blotting (approximately 200 nt; see Fig. 1a; Table S3 for details). The *sbrB* gene is situated between SA1173 (an unknown protein) and SA1174 (encoding *lexA*; see Fig. 1b). The *sbrB* also shares its intrinsic terminator with the upstream gene (SA1173) and is expected to be 149 nt long, corresponding to the size of the SbrB transcript observed in the northern blot analysis (approximately 150 nt; see Fig. 1b; Table S3 for details).

To analyze the SbrA and SbrB RNAs in more detail, we mapped their 5′-ends by primer extension analysis using total RNA purified from SH1000 and SH1000Δ*sigB* subjected to various σ^B activating stress conditions (i.e., KOH, $MnCl_2$, and NaCl stress). The results of these experiments are shown in Fig. 2a and b. In the wild-type strain, clear bands were detected for both transcripts following KOH and $MnCl_2$ stress, whereas no induction was observed for cells treated with 10% NaCl for 20 min, most likely due to the relatively mild and transient effect of NaCl on σ^B activity (Pane-Farre et al. 2006). No signals were observed in the Δ*sigB* mutant strain. In both cases, the 5′-ends of the transcripts map to the predicted σ^B-dependent transcription start sites for *sbrA* and *sbrB*, respectively.

sbrA and *sbrB* are present in various *S. aureus* isolates

BLAST searches for *sbrA* and *sbrB* homologs in other bacteria revealed that the genes are highly conserved among *S. aureus* strains. However, the expression of homologous genes among different *S. aureus* isolates may vary considerably. We therefore tested whether SbrA and SbrB could be detected in three additional strains of *S. aureus* (COL, Newman, and UAMS-1). As controls, we used their isogenic Δ*sigB* mutant strains. As shown in Fig. 3a, the expression of both transcripts was found to be induced by KOH stress in a strictly σ^B-dependent fashion in all *S. aureus* isolates tested. To further characterize the expression pattern of *sbrA* and *sbrB* in *S. aureus*, RNA levels were determined in COL, Newman, and SH1000 and their Δ*sigB* mutant strains at various growth time points during growth in rich medium. As shown in Fig. 3b–d, both RNAs are expressed in a strictly σ^B-dependent manner throughout growth. In all cases, the levels of the SbrA and SbrB RNAs increased significantly when wild-type cells entered the late exponential growth phase.

Expression of *sbrA* and *sbrB* does not depend on Hfq

Many sRNAs from other organisms depend on the RNA chaperone Hfq for stability and regulatory function (Valentin-Hansen et al. 2004; Aiba 2007). Although no such Hfq-dependent sRNAs have been identified so far in *S. aureus*, both SbrA and SbrB display some of the key features of Hfq-binding sRNAs (i.e., long AU-rich tracts next to stem-loop structures (Schumacher et al. 2002); see Fig. 1a and b for predicted secondary structures of the RNA molecules). We therefore asked whether Hfq might affect expression of *sbrA* or *sbrB*. To address this, we used *S. aureus* strain Newman and its isogenic Δ*hfq* mutant strain and tested the expression of SbrA and SbrB under different stress conditions. As a control, we included the Newman Δ*sigB* mutant. As shown in Fig. 3e, no differences were observed between the wild-type strain and Δ*hfq* mutant strain, suggesting that Hfq does not influence steady-state levels of either RNA. These results were confirmed by RNA half-life

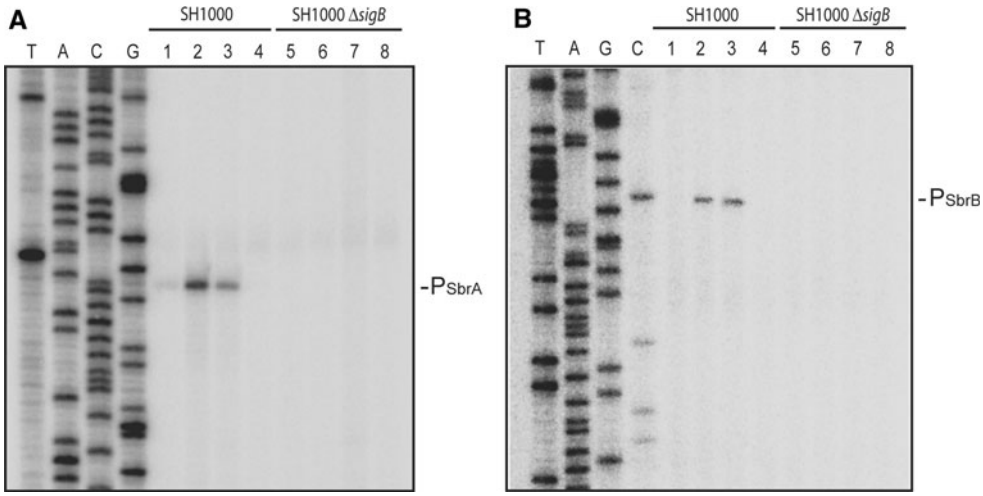

Fig. 2 Detection of 5′-ends of σ^B-regulated transcripts by primer extension analysis. *S. aureus* strain SH1000 and its isogenic Δ*sigB* strain were grown to early exponential phase at which point one of the following stress reagents were added: *lanes 1* and *5* no addition; *lanes 2* and *6* 30 mM KOH; *lanes 3* and *7* 1 mM $MnCl_2$; *lanes 4* and *8* 10% NaCl. The cells were stressed for 20 min. **a** Detection of 5′-end of *sbrA* RNA. **b** Detection of 5′-end of *sbrB* RNA

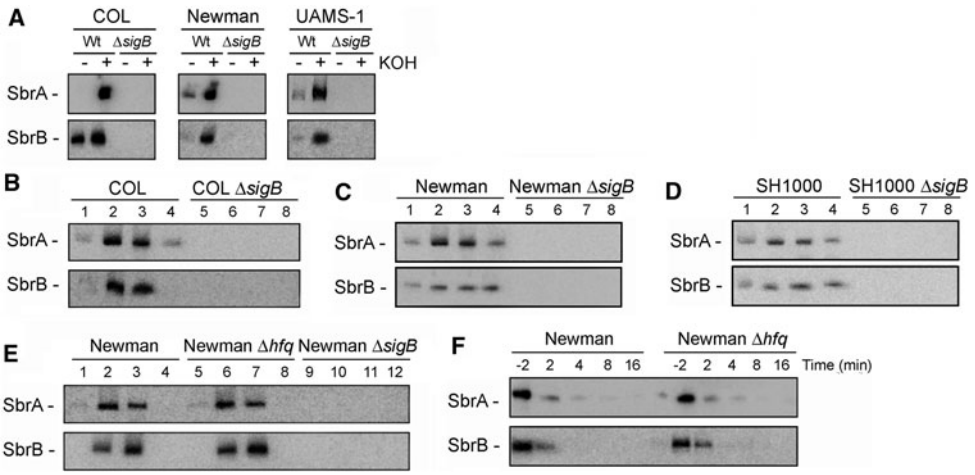

Fig. 3 *sbrA* and *sbrB* are expressed in different isolates of *S. aureus* and do not require Hfq for stability. **a** Northern blot showing induction of *sbrA* and *sbrB* in three different strains of *S. aureus*. **b**–**d** Northern blots showing expression of *sbrA* and *sbrB* in strains COL (**b**) Newman (**c**) and SH1000 (**d**) during growth. *Lanes 1* and *5* early exponential phase; *lanes 2* and *6* late exponential phase; *lanes 3* and *7* early stationary phase; *lanes 4* and *8* late stationary phase. **e** Northern blot showing induction of *sbrA* and *sbrB* in *S. aureus* strain Newman. Identical induction patterns are observed in wild-type and Δ*hfq* strains. *Lanes 1*, *5*, and *9* no stress; *lanes 2*, *6*, and *10* 30 mM KOH; *lanes 3*, *7*, and *11* 1 mM $MnCl_2$; *lanes 4*, *8*, and *12* 10% NaCl. The cells were induced for 20 min. **f** Half-life determination of *sbrA* and *sbrB* RNAs in wt and Δ*hfq* cells. Expression of both RNAs was induced by the addition of 30 mM KOH. After 10 min of induction, transcription was blocked by the addition of rifampicin, corresponding to time 0 min in the half-life experiment. Samples were drawn at the indicated timepoints relative to the addition of rifampicin

determinations, which did not reveal any difference in RNA decay after treatment with rifampicin (Fig. 3f). Similar results were obtained in the *S. aureus* COL background (data not shown). We conclude that, in vivo, Hfq does not seem to interact with the SbrA or SbrB RNAs in any way that alters their steady-state levels or stability. These results are in agreement with previous experiments, which have all failed to identify a role for Hfq in riboregulation in *S. aureus* (Bohn et al. 2007).

sbrA and *sbrB* encode two putative small basic peptides: SbpA and SbpB

Multiple alignments of *sbrA* and *sbrB* homologs from several *Staphylococci* (including *S. epidermidis*, *S. saprophyticus, S. warneri, S. hominis,* and *S. haemolyticus*) revealed that the IGRs at the corresponding genomic locations displayed very little sequence conservation at the DNA level (see supplementary materials Fig. S1). However, some regions, such as the putative σ^B consensus sites, appeared to be more conserved than others. In particular, we noticed AUG start codons as well as upstream putative ribosomal binding sites in SbrA and SbrB, which appeared to be conserved among several *Staphylococci*. We therefore searched for the presence of ORFs within these IGRs. In both regions, we detected short ORFs corresponding to highly basic peptides of 38 amino acids and 27 amino acids, respectively, in *S. aureus*. Moreover, the putative peptides were highly conserved among different *Staphylococci* (Fig. 4a, b). When scrutinizing other, more recent *S. aureus* genome annotations, we further found that small ORFs were predicted in the loci corresponding to *sbrA* and *sbrB*. In addition, the presence of a small ORF in *sbrB* was predicted in a recent study (Geissmann et al. 2009). Taken together, these observations indicate that both *sbrA* and *sbrB* contain small ORFs.

To test the presence of small ORFs in *sbrA* and *sbrB*, we constructed in-frame translational fusions of both ORFs to *lacZ* in the fusion vector pCK-*lacZ*. The resulting plasmids, named p*sbrA-lacZ* and p*sbrB-lacZ*, were introduced into *S. aureus* strains SH1000, Newman, and COL, as well as their isogenic Δ*sigB* mutant strains. The expression of the p*sbrA-lacZ* and p*sbrB-lacZ* was tested by performing β-galactosidase assays. The results of these experiments are presented in Fig. 4. Increasing levels of β-galactosidase activities were observed throughout growth in the wild-type strains containing p*sbrA-lacZ*, whereas no activity was recorded in the corresponding Δ*sigB* mutant background (Fig. 4c). These results suggest that the σ^B-dependent *sbrA* indeed contains an ORF which appears to be translated in all *S. aureus* wild-type strains tested here. Likewise, the p*sbrB-lacZ* fusion was found to be expressed in SH1000, whereas no β-galactosidase activity was recorded in SH1000 Δ*sigB* harboring p*sbrB-lacZ* (Fig. 4d). Surprisingly, the levels of β-galactosidase activity recorded for *S. aureus* strains Newman and COL containing p*sbrB-lacZ* corresponded to background levels, suggesting that the putative ORF in *sbrB* is only being translated in some

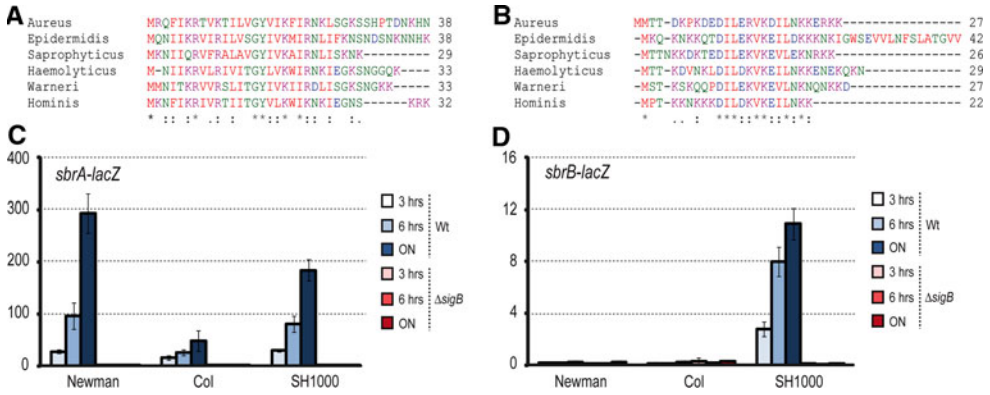

Fig. 4 *sbrA* and *sbrB* contain small open reading frames. Multiple alignments of the putative peptides ShpA (**a**) and ShpB (**b**) in different *Staphylococci*. Alignment was performed using ClustalW (ClustalW 2010). *β*-Galactosidase activity of translational fusions of *sbrA* (**c**) or *sbrB* (**d**) open reading frames to *lacZ*. Expression studies were carried out in three different *S. aureus* isolates (Newman, COL and SH1000; *blue bars*) and their isogenic Δ*sigB* mutant strains (*red bars*). Significant translation of *sbrB* was only observed in strain SH1000, whereas translation of *sbrA* was observed in all three wild-type *S. aureus* strains tested. In all cases tested, the expression of *sbrA-lacZ* and *sbrB-lacZ* depends on the presence of σ^B

S. aureus strains. We speculate that this discrepancy may be due to strain variations with respect to translation activating factors, or the presence of factors acting to repress translation of the ORF in *sbrB* in a subset of *S. aureus* strains. Analysis of cells subjected to KOH stress revealed similar results (data not shown). Based on these results, we conclude that *sbrA* and *sbrB* are likely to encode two small basic peptides, named SbpA and SbpB (*S*igmaB induced *B*asic *P*eptide *A* and *B*).

At present, the biological significance of *sbrA* and *sbrB* can only be speculated on. Both genes appear to encode small basic peptides with no apparent homologs found in the PDB database. Considering their small size, SbpA and SbpB may be signaling molecules similar to the quorum-sensing AgrD (Novick 2003) or the peptides could serve as binding partners for other proteins. In the latter case, such binding would presumably alter the function or activity of the partner protein. The primary sequences suggest that they are both highly basic and should therefore carry a net positive charge at physiological pH. Moreover, no hydrophobic patches or sequences corresponding to extracellular signaling peptides could be found, indicating that SbpA and SbpB are not membrane spanning nor are they exported outside the cell. Thus, a function similar to that described for the small hydrophobic IbsA-E peptides, which were found to be inserted into the membranes of *E. coli* thereby affecting membrane potential (Fozo et al. 2008), is unlikely. Alternatively, SbpA and SbpB may be RNA chaperones similar to what was postulated for three small basic peptides (named FbpA-C) in *Bacillus subtilis* (Gaballa et al. 2008). FbpA-C were found to be controlled by the ferric uptake repressor (Fur) and appeared to corroborate the functions of a non-coding RNA (FsrA). Similar to the findings in *S. aureus*, no role in riboregulation has so far been described for *hfq* in *B. subtilis,* and the authors therefore speculated that FbpA-C might act as RNA chaperones either alone or in heterooligomeric complexes dedicated to facilitate the function of FsrA. If SbpA and SbpB are in fact such dedicated RNA chaperones, this would seem to suggest the existence of a σ^B-controlled non-coding RNA as well.

SbrC is a small, non-coding RNA

The *sbrC* gene lies between SA0586 (encoding an IS1181 transposase) and SA0587 (encoding a protein, which is homologous to manganese-binding ABC transporters; see Fig. 1c). In this case, the predicted terminator of *sbrC* lies within the 3′ end of the downstream open reading frame (SA0587), and thus, the SbrC RNA could serve as a potential *cis*-acting antisense sRNA. The IGR between SA0586 and SA0587 was recently shown to encode another non-coding RNA (RsaC; [Geissmann et al. 2009)]. The *rsaC* gene lies in the opposite direction of *sbrC,* and the two genes do not overlap (Fig. 1c).

BLAST searches to identify *sbrC* homologs in relation to bacteria revealed that *sbrC* is conserved among all sequenced *S. aureus* strains but is absent from related *Staphylococci* such as *S. epidermidis*, *S. saprophyticus,* and *S. haemolyticus* (data not shown). Importantly, although these investigations did reveal potential ORFs within *sbrC*, none of these are preceded by a likely ribosomal binding site, indicating that the SbrC RNA is a genuine small, non-coding RNA. The expected length of SbrC is 310 nucleotides which correspond well with the result presented in Fig. 1c. Unfortunately, all our attempts to map the 5′- and 3′-ends of SbrC by primer extension analysis and RACE were unsuccessful, most likely due to relatively low levels

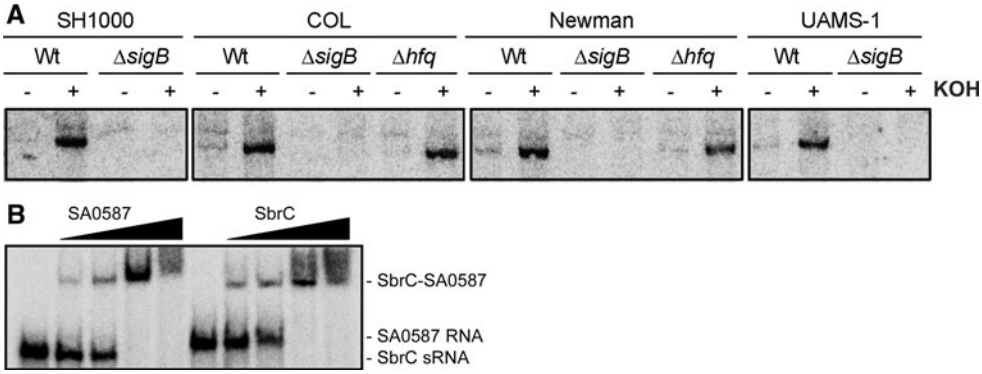

Fig. 5 The σ^B-regulated sRNA SbrC interacts with SA0587 in vitro. **a** Northern blotting showing expression of SbrC. Cells were grown to early exponential phase, and σ^B activity was induced by the addition of 30 mM KOH. The cells were stressed for 20 min. RNA was extracted from the indicated cells and subjected to northern blotting using a specific probe recognizing *sbrC* RNA. **b** Gelshift experiments showing that SbrC interacts with SA0587. In vitro transcribed SbrC (*left* part) or SA0587 (*right*), 5′-end labeled with ^{32}P, was incubated with or without increasing amounts of cold SA0587 (*left*) or SbrC RNA (*right*), respectively

of SbrC expression, or because of inhibitory secondary structures in SbrC that may prevent efficient ligation of the linker in RACE experiments.

To investigate the presence of SbrC in various *S. aureus* strains, we performed northern blotting using total RNA purified from SH1000, COL, Newman and UAMS-1 wild-type and Δ*sigB* mutant strains treated with KOH (Fig. 5a). As observed for *sbrA* and *sbrB*, the addition of KOH resulted in the induction of *sbrC* expression in a strictly σ^B-dependent manner in all wild-type strains tested. Furthermore, the levels of SbrC in COL Δ*hfq* and Newman Δ*hfq* were comparable to wild type, suggesting that the stability of SbrC is not affected by the RNA chaperone Hfq.

As mentioned, the *sbrC* gene overlaps the 3′-end of the SA0587 ORF, corresponding to *mntC* gene of the *mntABC* operon, by approximately 180 nucleotides, suggesting a possible *cis*-acting antisense regulatory mechanism. In support of this, gelshift assays with in vitro transcribed SbrC and truncated *mntC* RNA show that the two RNA species do indeed interact (Fig. 5b). The *mntABC* operon encodes an ABC-transporter dedicated to the uptake of manganese. Thus, the σ^B-dependent induction of *sbrC* could be a way for σ^B to affect manganese uptake during times of high extracellular manganese in *S. aureus*. The significance of such a regulatory circuit can only be speculated on. However, considering the effect of σ^B as well as proper manganese uptake on *S. aureus* virulence (Jonsson et al. 2004; Horsburgh et al. 2002b), it is likely to be important for effective host colonization by *S. aureus*.

Conclusion

We have identified three novel σ^B-dependent genes encoding one non-coding sRNA as well as two putative small basic peptides in the human pathogen *S. aureus*. σ^B-dependent expression of all three transcripts was confirmed in four different isolates of *S. aureus* (SH1000, COL, Newman and UAMS-1). The RNA chaperone Hfq did not affect steady-state levels or stability of either RNA suggesting, as reported previously (Bohn et al. 2007), that Hfq plays a very limited role in riboregulation in *S. aureus*.

In a recent study, two sRNAs named RsaA and RsaF were found to be under positive control by σ^B in *S. aureus* (Geissmann et al. 2009). Both *rsaA* and *rsaF* are preceded by putative σ^B-binding sites; however, these sites do not match perfectly with the σ^B consensus sequence (Geissmann et al. 2009). As a consequence of the very stringent search criteria employed in the present study, RsaA and RsaF were not among the sRNAs identified by us. These findings clearly demonstrate that in order to reveal the full repertoire of sRNAs in a given organism, it may be necessary to employ a variety of computational and experimental approaches.

The putative function of the σ^B-dependent sRNA and small ORFs is currently unknown. The basic nature of the two putative peptides suggest a possible role as RNA chaperones similar to what was proposed for three basic peptides in *B. subtilis* involved in modulating the iron sparring response (Gaballa et al. 2008). If SbpA and SbpB are indeed RNA chaperones, this would suggest the presence of a σ^B controlled non-coding RNA. A likely candidate would thus be SbrC, which appears to be a genuine σ^B controlled sRNA. Finally, we do not exclude the possibility that SbrA and SbrB could be dual-function RNAs, both acting as regulatory RNAs and encoding a protein product, in which case functional analyses becomes more complex. In this regard, we note that although SbrB is produced in all of the strains tested, the ORF within *sbrB* appears not to be translated in three out of four cases.

In either case, considering the fact that all three transcripts are strictly dependent on σ^B, we find it likely that the

RNAs and putative peptides encoded by *sbrA*, *sbrB,* and *sbrC* are involved in *S. aureus* stress response, antibiotic resistance, and/or virulence. Future studies in our laboratory will focus on revealing the physiological role and mode of action of small σ^B-dependent RNAs and peptides in *S. aureus*.

Experimental procedures

Growth strains and media

The bacterial strains used in the present study are listed in Table 2. Where indicated, isogenic Δ*sigB* or Δ*hfq* strains were used. Since foreign DNA is subject to degradation by resident deoxy-ribonucleases in *S. aureus*, plasmids were initially cloned in the restriction-deficient strain RN4220 and then transferred to relevant laboratory strains by transduction using φ11 as described by (McNamara and Iandolo 1998). Generalized transduction with φ11 was also used to move the rsbVW-sigB::erm mutation from the Newman strain (Kullik et al. 1998) into SH1000. For cloning purposes, *E. coli* TOP10 (Invitrogen) was used. *S. aureus* strains were grown in Luria–Bertani (LB), or Tryptic Soy Broth (TSB, Oxoid), when indicated. *E. coli* strains were grown in LB. When appropriate, kanamycin was supplemented to a final concentration of 50 μg/ml.

Prediction of putative intergenic σ^B-regulated sRNAs

For the prediction of putative intergenic σ^B-controlled transcripts, we initially searched for the presence of σ^B consensus sites [GWWT_$N_{14–17}$_GGGWWW, (Pane-Farre et al. 2006)] within the IGRs of the *S. aureus* N315 genome using the AureoList database (AureoList 2010). We have previously used this approach successfully to identify a novel σ^B-controlled sRNA in *L. monocytogenes* (Nielsen et al. 2008). The consensus sequence chosen was based on the results of two previous studies of the σ^B regulon in *S. aureus* (Bischoff et al. 2004; Pane-Farre et al. 2006). Both studies conclude that the σ^B consensus binding site in S. *aureus* closely resembles that found in *B. subtilis*, GttTww-$N_{12–15}$-gGgwAw (Petersohn et al. 1999). We have employed a strict interpretation of this to minimize the number of false positives. The initial search yielded 133 putative σ^B promoter sites (see supplementary material Table S1). Promoter sites located too close to and in the same direction of downstream ORFs were discarded yielding 61 potential sites (see supplementary material Table S2). In validation of our approach, we note that approximately 60% of the disregarded promoter sites corresponded to genes that have previously been reported as being positively regulated by σ^B (Bischoff et al. 2004). We next searched for the presence of an intrinsic terminator (here defined as an inverted repeat with a ΔG of no more than −8.5 kcal/mol followed by at least 4 Ts) no longer than 500 nucleotides downstream of a potential σ^B consensus site. This final search resulted in seven putative σ^B-regulated sRNA genes (see supplementary material Table S3). Two of the seven genes were immediately discarded. One candidate contained an annotated ORF (SA0039, 65 amino acids) and was therefore not studied any further. Another candidate corresponds to a likely transcriptional attenuator regulating expression of SA0347, which will not be considered in the present study. Thus, our bioinformatics approach resulted in the prediction of five putative σ^B-regulated genes

Table 2 Bacterial strains used in the present study

Strain	Description	Source
E. coli		
Top10	Transformation optimized strain for cloning	Invitrogen
S. aureus		
RN4220	Restriction-deficient transformation recipient	Kreiswirth et al. (1983)
SH1000	Functional *rsbU* derivative of 8325-4, *rsbU*$^+$	Horsburgh et al. (2002a)
SH1000 Δ*sigB*	SH1000 single deletion of *sigB* operon, ERM^R	This work
Newman	Clinical isolate (ATCC 25904), *rsbU*$^+$	Duthie and Lorenz (1952)
Newman Δ*sigB*	Newman single deletion of *sigB* operon, ERM^R	Kullik et al. (1998)
Newman Δ*hfq*	Newman single deletion of *hfq*	Bohn et al. (2007)
COL	Methicillin-resistant clinical isolate	de Lencastre and Tomasz (1994)
COL Δ*sigB*	COL single deletion of *sigB* operon, ERM^R	Kullik et al. (1998)
COL Δ*hfq*	COL single deletion of *hfq*	Bohn et al. (2007)
UAMS-1	Clinical osteomyelitis isolate, *rsbU*$^+$	Gillaspy et al. (1995)
UAMS-1 Δ*sigB*	Single deletion of *sigB* operon, Tet^R	Cassat et al. (2006)
ATCC 33591	Methicillin-resistant clinical isolate	Klitgaard et al. (2008)

Table 3 DNA oligos used in this study

Name	DNA sequence[a,b]	Use
T7_SbrA_NB F	GTGTAATACGACTCACTATAGGGAGTGGCGCTGTATTTTTAATTATGTTTATTATCTGTCGG	NB[c] of *sbrA*
SbrA_NB R	GTAATTAAATTTATTCGAAATAAACTTTCAGGTAAATCATCACATCCGACAGATAATAAAC	NB[c] of *sbrA*
T7_SbrB_NB F	GTGTAATACGACTCACTATAGGGGAGCGAGCCATTATTATTTTTTTCTTTCTTTTTTATTAAG	NB[c] of *sbrB*
SbrB_NB R	CTGTAAGGAAGTAGATAAACATGATGAC	NB[c] of *sbrB*
SbrC_NB	GTCGAAGGCTAATAGTCCCATATCGTGCG	NB[d] of *sbrC*
SbrD_NB	CCGAAAGCCTGAATGCAAGTCTTGATTAAATC	NB[d] of *sbrD*
SbrE_NB	GCGTACTTGGCTCAAAACTTTTACTTTCTCATCTATTT	NB[d] of *sbrE*
*Eco*RI_SbrA_PE_F	GGGGGAATTCCTGGACAGGTATCCCTACC	PE of *sbrA*, Fwd primer *sbrA-lacZ*
*Bam*HI_SbrA_PE_R	GGGGGGATCCGTCGGATGTGATGATTTACCTG	PE of *sbrA*
*Bam*HI_SbrA_pCK_R	GGGGGGATCCGGATGTGATGATTTACCTG	In-frame *sbrA-lacZ* fusion
*Eco*RI_SbrB_PE_F	GGGGGAATTCCTCAGCCGGAGAGAATTTAGC	PE of *sbrB*, Fwd primer *sbpA-lacZ*
*Bam*HI_SbrB_PE_R	GGGGGGATCCCAGGAGCGAGCCATTATTATTTTT	PE of *sbrB*
*Bam*HI_SbrB_pCK_R	GGGGGGATCCGCATCTTTTGGTTTATCTG	In-frame *sbpB-lacZ* fusion
- 40 primer	GTTTTCCCAGTCACGACGTTGTAAAACGACGG	Sequencing of pCK-*lac* constructs
V-lac 1	GTTGAATAACACTTATTCCTATC	Sequencing of pCK-*lac* constructs
*Eco*RI_SbrC_PE_F		PE of *sbrC*
*Bam*HI_SbrC_PE_R	TATTTTAGGCTACACATCAACATAACAA	PE of *sbrC*
T7_EcoRI_SA0587_F	TTAAGAATTC_TAATACGACTCACTATA_GGGAAATTAACACTGAAAAACAAG	In vitro SA0587
BamHI_SA0587_R	TTAAGGAT_CCTAAAATATTGGAGATACC	In vitro SA0587
T7_EcoRI_sbrC_F	TTAAGAATTC_TAATACGACTCACTATAGG_TTTAACGCACGATATGGGACTATTAG	In vitro SbrC
BamHI_sbrC_R	TTAAGGATCC_AAATTAAAACACTTATTAGTAGAAAC	In vitro SbrC

[a] T7-promoter sites are underlined

[b] Restriction enzyme cleavage sites are indicated by a gray shade

[c] DNA oligos used for PCR to generate template for in vitro transcribed northern probe

[d] DNA oligo used directly as northern probe

within the IGR of *S. aureus* strain N315, none of which contain annotated ORFs.

RNA work

For the detection of *sbrA-E* RNAs, *S. aureus* strains were grown to early exponential phase (OD_{600} = 0.4) at which point the cells were stressed by the addition of 30 mM KOH, 1 mM $MnCl_2$ or 10% NaCl (final concentrations). Ten-milliliter samples were collected at time 0 min (control samples) and at the indicated time points. Cells were re-suspended in Tri Reagent (MRCGENE) as described by the manufacturer and lysed using the Fast-Prep instrument (2 × 40 s at maximum speed), and RNA was extracted as described previously for *L. monocytogenes* (Nielsen et al. 2010). The integrity of the RNA was verified by agarose gel electrophoresis, and the purity and concentration were determined using a NanoDrop 2000. Equal amounts of total RNA (15 µg, containing equal amounts of 23S and 16S rRNA) and the RNA size marker RNA Century™ (Ambion) were separated on a 6% denaturing polyacrylamide gel and subsequently transferred to a Zeta probe membrane by semi-dry electroblotting. The membranes were hybridized in PerfectHyb (Sigma) to either a 5′-end labeled DNA oligo or a 5′-end labeled in vitro transcribed RNA (prepared with T7-RNA polymerase) complementary to each RNA (see Table 3 for details on DNA oligos). Following overnight hybridization, membranes were washed according to the manufactures instructions. The results were visualized by autoradiography and phosphoimaging using a Typhoon scanner. To minimize the risk of technical or biological errors, the experiments were repeated at least three times, with identical outcomes.

Primer extension was performed as described previously (Nielsen et al. 2010). Briefly, RNA was collected from *S. aureus* cells subjected to different stress forms as indicated in Fig. 2. cDNA synthesis was initiated with 15 µg total RNA as template using the reverse PE primers shown in Table 3. In parallel, a sequencing reaction was run for each sRNA tested. A PCR fragment prepared with the primers listed in Table 3 was used as template in the sequencing reaction.

The interaction between SbrC and SA0587 was analyzed by gelshift assays. RNAs corresponding to the predicted SbrC and a truncated version of SA0587 (335 nt) was prepared by in vitro transcription and gelshifts were performed as previously described (Nielsen et al. 2010). Briefly, 40 fmol of in vitro transcribed, 5′-end labeled SbrC or SA0587, was incubated with or without increasing amounts of cold SA0587 or SbrC RNA respectively (0-, 5-, 10-, 20-, and 50-fold molar excess). As a non-specific competitor, 1 µg yeast tRNA was added.

Construction of translational lacZ fusions and β-galactosidase assay

In-frame translational fusions of *sbrA* and *sbrB* to *lacZ* were constructed as follows: DNA fragments containing the likely promoter region as well as the first amino acids of the putative peptide encoded by *sbrA* or *sbrB* were generated by PCR using the primers listed in Table 3. The resulting DNA fragments were cleaved by *Eco*RI and *Bam*HI and ligated into the corresponding *Eco*RI-*Bam*HI site in the translational *lacZ* fusion vector, pCK-*lac* (a derivative of the transcriptional *lacZ* fusion vector pTCV-*lac* (Poyart and Trieu-Cuot 1997); B. H. Kallipolitis, unpublished) resulting in plasmids p*sbrA-lacZ* and p*sbrB-lacZ*. Plasmids were used to transform competent *S. aureus* cells.

Plasmids pCK-*lac*, p*sbrA-lacZ,* and p*sbrB-lacZ* were first electroporated into competent RN4220 cells, which were prepared by inoculating pre-warmed TSB with cells from an overnight culture. The culture was grown at 37°C to $OD_{600} \approx 0.5$ at which point cells were harvested and washed four times in ice-cold sterile 0.5 M sucrose with 0.5, 0.25, 0.125, and 0.0625 times the original culture volume. Following the second wash, the re-suspended cells were incubated on ice for 2 h before proceeding. After the wash, cells were re-suspended into ice-cold, filter-sterilized 10% glycerol, and stored at −80°C until use. Electroporation was performed as described by (Kraemer and Iandolo 1990). Following electroporation, the *lacZ* fusions were transduced into wild-type and Δ*sigB* mutant strains (SH1000, Newman and COL) as described by (McNamara and Iandolo 1998) using φ11.

For measurement of *sbrA-lacZ* and *sbrB-lacZ* translation, *S. aureus* strains SH1000, Newman and COL (wild-type and isogenic Δ*sigB*) carrying the p*sbrA-lacZ* and p*sbrB-lacZ* were grown in TSB. One-milliliter aliquots were collected after 1, 3, 6, and 24 h of growth. Subsequently, cells were permeabilized by treatment with 50 µg/ml lysostaphin in 50 mM Tris pH 8 at 37°C with gentle shaking for 2 h, and β-galactosidase activities determined as described previously (Christiansen et al. 2004).

Acknowledgments We are grateful to Markus Bischoff (University of Saarland Hospital) and Mark Smeltzer (University of Arkansas) for the supply of strains. This work was supported by The Danish Council for Independent Research/Natural Sciences and Lundbeckfonden.

References

Aiba H (2007) Mechanism of RNA silencing by Hfq-binding small RNAs. Curr Opin Microbiol 10:134–139

AureoList (2010) AureoList. World Wide Web. http://genolist.pasteur.fr/AureoList/

Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, Berger-Bachi B, Projan S (2004) Microarray-based analysis of

the *Staphylococcus aureus* sigmaB regulon. J Bacteriol 186:4085–4099

Bohn C, Rigoulay C, Bouloc P (2007) No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. BMC Microbiol 7:10

Bohn C, Rigoulay C, Chabelskaya S, Sharma CM, Marchais A, Skorski P, Borezee-Durant E, Barbet R, Jacquet E, Jacq A, Gautheret D, Felden B, Vogel J, Bouloc P (2010) Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. Nucleic Acids Res. doi:10.1093/nar/gkq462

Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M, Chevalier C, Helfer AC, Benito Y, Jacquier A, Gaspin C, Vandenesch F, Romby P (2007) Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev 21:1353–1366

Cassat J, Dunman PM, Murphy E, Projan SJ, Beenken KE, Palm KJ, Yang SJ, Rice KC, Bayles KW, Smeltzer MS (2006) Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic agr and sarA mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology 152:3075–3090

Christiansen JK, Larsen MH, Ingmer H, Sogaard-Andersen L, Kallipolitis BH (2004) The RNA-binding protein Hfq of Listeria monocytogenes: role in stress tolerance and virulence. J Bacteriol 186:3355–3362

ClustalW (2010) ClustalW. World Wide Web. http://www.ebi.ac.uk/Tools/clustalw2/index.html

de Lencastre H, Tomasz A (1994) Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 38:2590–2598

Duthie ES, Lorenz LL (1952) Staphylococcal coagulase; mode of action and antigenicity. J Gen Microbiol 6:95–107

Fozo EM, Kawano M, Fontaine F, Kaya Y, Mendieta KS, Jones KL, Ocampo A, Rudd KE, Storz G (2008) Repression of small toxic protein synthesis by the Sib and OhsC small RNAs. Mol Microbiol 70:1076–1093

Gaballa A, Antelmann H, Aguilar C, Khakh SK, Song KB, Smaldone GT, Helmann JD (2008) The Bacillus subtilis iron-sparing response is mediated by a Fur-regulated small RNA and three small, basic proteins. Proc Natl Acad Sci USA 105:11927–11932

Geisinger E, Adhikari RP, Jin R, Ross HF, Novick RP (2006) Inhibition of rot translation by RNAIII, a key feature of agr function. Mol Microbiol 61:1038–1048

Geissmann T, Chevalier C, Cros MJ, Boisset S, Fechter P, Noirot C, Schrenzel J, Francois P, Vandenesch F, Gaspin C, Romby P (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. Nucleic Acids Res 37:7239–7257

Gertz S, Engelmann S, Schmid R, Ziebandt AK, Tischer K, Scharf C, Hacker J, Hecker M (2000) Characterization of the sigma(B) regulon in *Staphylococcus aureus*. J Bacteriol 182:6983–6991

Giachino P, Engelmann S, Bischoff M (2001) Sigma(B) activity depends on RsbU in *Staphylococcus aureus*. J Bacteriol 183:1843–1852

Gillaspy AF, Hickmon SG, Skinner RA, Thomas JR, Nelson CL, Smeltzer MS (1995) Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. Infect Immun 63:3373–3380

Gimpel M, Heidrich N, Mader U, Krugel H, Brantl S (2010) A dual-function sRNA from B. subtilis: SR1 acts as a peptide encoding mRNA on the gapA operon. Mol Microbiol 76:990–1009

Hemm MR, Paul BJ, Schneider TD, Storz G, Rudd KE (2008) Small membrane proteins found by comparative genomics and ribosome binding site models. Mol Microbiol 70:1487–1501

Hemm MR, Paul BJ, Miranda-Rios J, Zhang A, Soltanzad N, Storz G (2010) Small stress response proteins in *Escherichia coli*: proteins missed by classical proteomic studies. J Bacteriol 192:46–58

Hobbs EC, Astarita JL, Storz G (2010) Small RNAs and small proteins involved in resistance to cell envelope stress and acid shock in *Escherichia coli*: analysis of a bar-coded mutant collection. J Bacteriol 192:59–67

Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ (2002a) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol 184:5457–5467

Horsburgh MJ, Wharton SJ, Cox AG, Ingham E, Peacock S, Foster SJ (2002b) MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. Mol Microbiol 44:1269–1286

Jonsson IM, Arvidson S, Foster S, Tarkowski A (2004) Sigma factor B and RsbU are required for virulence in *Staphylococcus aureus*-induced arthritis and sepsis. Infect Immun 72:6106–6111

Klitgaard JK, Skov MN, Kallipolitis BH, Kolmos HJ (2008) Reversal of methicillin resistance in *Staphylococcus aureus* by thioridazine. J Antimicrob Chemother 62:1215–1221

Kraemer GR, Iandolo JJ (1990) High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr Microbiol 21:373–376

Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305:709–712

Kullik I, Giachino P (1997) The alternative sigma factor sigmaB in *Staphylococcus aureus*: regulation of the sigB operon in response to growth phase and heat shock. Arch Microbiol 167:151–159

Kullik I, Giachino P, Fuchs T (1998) Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J Bacteriol 180:4814–4820

Lorenz U, Huttinger C, Schafer T, Ziebuhr W, Thiede A, Hacker J, Engelmann S, Hecker M, Ohlsen K (2008) The alternative sigma factor sigma B of *Staphylococcus aureus* modulates virulence in experimental central venous catheter-related infections. Microbes Infect 10:217–223

Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339:520–532

McNamara PJ, Iandolo JJ (1998) Genetic instability of the global regulator agr explains the phenotype of the xpr mutation in *Staphylococcus aureus* KSI9051. J Bacteriol 180:2609–2615

MFOLDi (2010) MFOLD. World Wide Web. http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi

Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, Takeyasu K, Ohta T (2003) A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells 8:699–712

Nielsen JS, Olsen AS, Bonde M, Valentin-Hansen P, Kallipolitis BH (2008) Identification of a sigma B-dependent small noncoding RNA in Listeria monocytogenes. J Bacteriol 190:6264–6270

Nielsen JS, Lei LK, Ebersbach T, Olsen AS, Klitgaard JK, Valentin-Hansen P, Kallipolitis BH (2010) Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in Listeria monocytogenes. Nucleic Acids Res 38:907–919

Novick RP (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol 48:1429–1449

Pane-Farre J, Jonas B, Forstner K, Engelmann S, Hecker M (2006) The sigmaB regulon in *Staphylococcus aureus* and its regulation. Int J Med Microbiol 296:237–258

Petersohn A, Bernhardt J, Gerth U, Hoper D, Koburger T, Volker U, Hecker M (1999) Identification of sigma(B)-dependent genes in *Bacillus subtilis* using a promoter consensus-directed search and oligonucleotide hybridization. J Bacteriol 181: 5718–5724

Pichon C, Felden B (2005) Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. Proc Natl Acad Sci USA 102:14249–14254

Poyart C, Trieu-Cuot P (1997) A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. FEMS Microbiol Lett 156:193–198

Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG (2002) Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. EMBO J 21:3546–3556

Sharma CM, Vogel J (2009) Experimental approaches for the discovery and characterization of regulatory small RNA. Curr Opin Microbiol 12:536–546

Shaw LN, Lindholm C, Prajsnar TK, Miller HK, Brown MC, Golonka E, Stewart GC, Tarkowski A, Potempa J (2008) Identification and characterization of sigma, a novel component of the *Staphylococcus aureus* stress and virulence responses. PLoS One 3:e3844

Valentin-Hansen P, Eriksen M, Udesen C (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. Mol Microbiol 51:1525–1533

Wadler CS, Vanderpool CK (2007) A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. Proc Natl Acad Sci USA 104:20454–20459

Waters LS, Storz G (2009) Regulatory RNAs in bacteria. Cell 136:615–628

Wu S, de Lencastre H, Tomasz A (1996) Sigma-B, a putative operon encoding alternate sigma factor of Staphylococcus aureus RNA polymerase: molecular cloning and DNA sequencing. J Bacteriol 178:6036–6042