Opposing roles of $\sigma^\text{B}$ and $\sigma^\text{B}$-controlled SpoVG in the global regulation of esxA in Staphylococcus aureus

Bettina Schulthess1*, Dominik A Bloes1,2 and Brigitte Berger-Bächi1

Abstract

Background: The production of virulence factors in Staphylococcus aureus is tightly controlled by a complex web of interacting regulators. EsxA is one of the virulence factors that are excreted by the specialized, type VII-like Ess secretion system of S. aureus. The esxA gene is part of the $\sigma^\text{B}$-dependent SpoVG subregulon. However, the mode of action of SpoVG and its impact on other global regulators acting on esxA transcription is as yet unknown.

Results: We demonstrate that the transcription of esxA is controlled by a regulatory cascade involving downstream $\sigma^\text{B}$-dependent regulatory elements, including the staphylococcal accessory regulator SarA, the ArlRS two-component system and SpoVG. The esxA gene, preceding the ess gene cluster, was shown to form a monocistronic transcript that is driven by a $\sigma^\text{A}$ promoter, whereas a putative $\sigma^\text{B}$ promoter identified upstream of the $\sigma^\text{A}$ promoter was shown to be inactive. Transcription of esxA was strongly upregulated upon either sarA or sigB inactivation, but decreased in agr, arlR and spoVG single mutants, suggesting that agr, ArlR and SpoVG are able to increase esxA transcription and relieve the repressing effect of the $\sigma^\text{B}$-controlled SarA on esxA.

Conclusion: SpoVG is a $\sigma^\text{B}$-dependent element that fine-tunes the expression of esxA by counteracting the $\sigma^\text{B}$-induced repressing activity of the transcriptional regulator SarA and activates esxA transcription.
essABC, esaABC and esaD [14,15] and secretes proteins with a size of approximately 100 amino acids containing a helical structure and a conserved Trp-Xaa-Gly (WXG) motif [16]. Three proteins were so far shown to be exported by the staphylococcal Ess system, two WXG100 family proteins, EssA and EssB, and the non-WXG100 substrate EsaC [14,17]. All three proteins act as pathogenicity factors in a murine model of staphylococcal blood-borne dissemination and abscess formation [14,17]. The actual role of EssA, EssB and EsaC remains unclear. Structural analysis of EssA suggests a role as transport module or chaperone to assist export of proteins by the Ess secretion pathway rather than being an effector protein itself [18]. The essA gene seems to be under complex control. Besides being upregulated by SpoVG [10], essA was found to be upregulated by ArlR [19]. The two-component system ArlRS [19,20] itself is activated in an indirect way by σB in strain Newman [3,9], adding a further level of complexity in the regulation of essA.

This study analyses the transcriptional control of essA by σB and the σB-dependent regulatory elements SarA, ArlR, RNAIII and SpoVG.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids are listed in Table 1. Bacteria were grown on Luria Bertani (LB) agar (Becton Dickinson, Franklin Lakes, NJ, USA) or in LB broth with shaking (180 rpm) at 37°C in a flask to medium ratio 5:1. Where required, media were supplemented with 100 μg ml⁻¹ ampicillin, 20 μg ml⁻¹ chloramphenicol, 10 μg ml⁻¹ erythromycin, or 10 μg ml⁻¹ tetracycline.

Molecular biological methods

General molecular biology techniques were performed according to standard protocols [32,33]. Sequencing was done using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed with the Lasergene software package (DNASTAR, Inc., Madison, WI, USA).

Construction of ΔessA mutants

The markerless deletion of essA (nwmn_0219) in strains Newman, BB1002 and NM143 was constructed using the counter selection system of pKOR1 as described by Bae et al. [28], using primer pairs oBS43/oBS44 and oBS45/oBS46 (Table 2) to amplify sequences framing essA. Correct deletion of essA in BS304, BS307 and BS308, respectively, was confirmed by sequencing and Southern blot analysis, and the absence of major rearrangements by pulsed-field gel electrophoresis [34].

Construction of BS309 and BS310

The Newman sarA mutant BS309 and the Newman agr mutant BS310 were constructed by transducing the ermB-tagged sarA mutation of NM520 [38], and the ermB-tagged agr mutation of NM521 [38] respectively, into Newman and selecting for erythromycin resistance. Correct inactivation of the genes was confirmed by sequencing and Southern blot analysis.

Plasmid construction

For the construction of promoter-lacZ reporter fusions, DNA fragments covering the yabJ or essA promoter of strain Newman were amplified using primer pairs yab-prom-bam-f/yab-prom-xho-r and Pnwmn0219F/Pnwmn0219R-xho (Table 2), respectively. The PCR products were digested with BamHI and XhoI and ligated into promoter probe plasmid pSB40N [29] upstream of the lacZα reporter gene to obtain pyaAβp and pexAxA.

For the construction of pexxAβp-luc⁺, the essA promoter region of strain Newman was amplified by PCR using primer pair Pnwmn0219F-hind/Pnwmn0219R (Table 1). The resulting PCR product was HindIII/NcoI-digested and cloned into pSB40N upstream of the luciferase reporter gene luc⁺. The essA promoter-luc⁺ fusion of the resulting plasmid was amplified using the primers Pnwmn0219F-hind/pSP-luc⁺ upstream of the luciferase reporter gene luc⁺. The corresponding DNA fragments were amplified with primer pairs oBS49/oBS53 and oBS51/oBS54 (Table 2) from pexxAβp-luc⁺ and religated.

All plasmids constructs were confirmed by sequence analyses.

Northern blot analysis

Overnight cultures were diluted 1:100 into LB, grown for 2 h, and then used to inoculate 100 ml of pre-warmed LB to an optical density of 600 nm [OD₆₀₀ₙ₉] of 0.05. Cell samples were taken at the time points indicated, centrifuged at 12,000 × g and 4°C for 2 min, the pellets were snap-frozen in liquid nitrogen. Total RNA was isolated according to Cheung et al. [39]. RNA samples (8 μg) were separated in a 1.5% agarose gel containing 20 mM guanidine thiocyanate in 1 × Tris-borate-EDTA buffer [40]. RNA transfer and detection were performed as previously described [41,42]. Digoxigenin (DIG) labelled probes were amplified using the PCR DIG Probe synthesis kit (Roche, Basel, Switzerland). The primer pairs used for amplification of the essA, spoVG, asp23, arlR, sarA and RNAIII probes are listed in Table 2.
Primer extension

RNA was extracted from LR15 cultures that were grown to OD600 nm 2.0, as described by Cheung et al. [39]. Primer extension reactions were performed using 20 μg of total RNA and 3 pmol of the 5'-biotin-labelled primers pe_esxA_1 and pe_esxA_2 (Table 2) using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturers instructions. Sequencing reactions were performed using the Thermo Sequenase Cycle Sequencing Kit (USB Corporation, Cleveland, OH, USA) and template DNA amplified with primers Pnmmn0219F and esxA_term-r from Newman genomic DNA. The Biotin Chromogenic Detection Kit (Fermentas, Burlington, Ontario, Canada) was used for biotin detection.

Table 1 Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype; phenotype | Reference or source |
|-------------------|-----------------------------|---------------------|
| **S. aureus**     |                             |                     |
| Newman            | Clinical isolate, ATCC 25904, natural saeS constitutive mutant | [21,22]             |
| BS304             | Newman ΔaesA, markerless deletion | This study         |
| SM148             | Newman Δ(yabJ-spoVG)-ermB; Em' | [8]                 |
| IK184             | Newman Δ(rsbUVW-sigB)-ermB; Em' | [23]                |
| MS64              | Newman sigB1(Am); Tc'       | [24]                |
| SM99              | Newman Δxdr:cat; Cm'        | [9]                 |
| BS310             | Newman Δagr:ermB; Em'       | This study          |
| KS186             | Newman Δagr:tet(M); Tc'     | [25]                |
| BS309             | Newman ΔanA:ermB; Em'       | This study          |
| LR15              | Newman ΔanA:tet(U); Tc'     | L. Reutimann        |
| BB1002            | Newman mec, MRSA derivative, Mc' | [26]              |
| BS307             | BS1002 ΔesxA, markerless deletion | This study        |
| NM143             | Newman GISA derivative, in vitro selected mutant; Te' | [27]               |
| BS308             | NM143 ΔesxA, markerless deletion | This study       |
| **E. coli**       |                             |                     |
| DH5α              | F-Φ80d/acZ ΔM15 recA1       | Invitrogen          |
| **Plasmids**      |                             |                     |
| pKOR1             | *E. coli-S. aureus* shuttle vector for markerless deletions using the counter selection system | [28]           |
| pAC7              | Expression plasmid containing the Pmkl promoter and the araC gene; Cm' | [29]     |
| pAC7-sigB         | pAC7 with a 0.75 kb fragment containing the gene sigB from S. aureus Col; Cm' | [30]        |
| pBus1             | *E. coli-S. aureus* shuttle plasmid with multicloning site from pBluescript II SK (Stratagene) and the rrnT14 terminator sequence from pLL2443; Tc' | [31]    |
| ppyab1            | pBus1 containing a bacA promoter-yab1 ORF fusion construct; Tc' | [10]    |
| pspoVG            | pBus1 containing a bacA promoter-spoVG ORF fusion construct; Tc' | [10]    |
| ppyab1spoVG       | pBus1 containing a bacA promoter-yab1-spoVG operon fusion construct; Tc' | [10]    |
| pSp-luc\*         | Firefly luciferase cassette vector; Ap' | Promega         |
| pesxAprom-luc\*  | pBus1 containing an esxA-luc\* fusion fragment; Tc' | This study |
| pesxAprom\*       | pesxA-luc\* with deletion of the σA promoter | This study |
| pesxAprom\*       | pesxA-luc\* with deletion of the σA promoter | This study |
| pSB40N            | Promoter probe plasmid; Ap' | [29]            |
| pasp23p           | pSB40N with a 0.6 kb fragment covering the asp23 promoter region fused to the reporter gene lacZα; Ap' | [30] |
| pesxA             | pSB40N with a 0.3 kb fragment covering the esxA promoter region fused to the reporter gene lacZα; Ap' | This study |
| ppyab1            | pSB40N with a 0.4 kb fragment covering the yab1 promoter region fused to the reporter gene lacZα; Ap' | This study |
| pSTM07            | pSB40N with a 0.37 kb fragment covering the capA promoter region fused to the reporter gene lacZα; Ap' | [9] |

Abbreviations are as follows: Ap', ampicillin resistant; Cm', chloramphenicol resistant; Em', erythromycin resistant; Mc', methicillin resistant; Tc', tetracycline resistant; Te', teicoplanin resistant.
Two-plasmid testing
Testing of the interaction of S. aureus promoters with E. coli RNA polymerase containing S. aureus Cap was done essentially as described earlier [30]. The promoter-reporter plasmids pasp23p (asp23 promoter); pyabJp (yabJ promoter); psexap (sexA promoter); and pSTM07 (capA promoter); or the empty plasmid pSB40N, were transformed into E. coli DH5α containing either pAC7-sigB or pAC7. The color production of the clones was analyzed on LBACX-ARA plates (LB agar containing 5 mg ml⁻¹ lactose; 100 μg ml⁻¹ ampicillin; 40 μg ml⁻¹ chloramphenicol; 20 μg ml⁻¹ X-Gal (5-bromo-4-choro-3-indoly-D-galactopyranoside) and 2 μg ml⁻¹ arabinose) [29].

Luciferase assay
Luciferase activity was measured as described earlier [3] using the luciferase assay substrate and a Turner Designs TD-20/20 luminometer (Promega).

Table 2 Oligonucleotide primers used in this study

| Primer name | Sequence (5’-3’)a | reference |
|-------------|-------------------|-----------|
| oBS43       | GGGGACAAGTTTTTGATCAAAAAAGCAGGCTacgtttatcaaagacatacc | This study |
| oBS44       | gggggtaccacatgaataacctctggata | This study |
| oBS4S       | gggtaccagcattctgaaatgccaag | This study |
| oBS46       | GGGGACCCTTTTGTACAAAAGGCTGTTtcatctgctgtatttg | This study |

**DIG probes**

- Nxwmn0219-DIG-f: tccagagg1aactcagaaace [10]
- Nxwmn0219-DIG-r: ctttcgttaggcggcatca [10]
- oSTM29 (spoVG) ggcgtcgaaccttgcaaaagttagatcgc [9]
- oSTM34 (spoVG) gcgggaacctcgattcttcatcattagatg [8]
- SasaAF: aagggagttaaaacatg [35]
- SasaAF: ctcgactaaatgatcgg [35]
- RNAll+ gtagatgaaaatgatgag [3]
- RNAll gtagatggtgctagctg [3]
- arIRsprobe+ tcgtatcacatacacaacgc [36]
- arIRsprobe gtagatgatgagaagtgcgcg [36]
- SAAtp23+ atgaactgtgatagaacaaagc [37]
- SAAtp23 ttgtaacactctttctgg [37]

**plasmid construction**

- Pnwmn0219F: tgggagatcctgacacggtttgtcgtg [This study]
- Pnwmn0219R-xho: tgcctcgaagcctaatgatattttttttaag [This study]
- yab-prom-bam-f: gcgggaacctctgtaaatatattaattacc [This study]
- yab-prom-xho-r: gcgtcgagatcctaatcctttatgcgaaac [This study]
- Pnwmn0219F-hind: tgcgaacctgacacggtttgtcgtg [This study]
- Pnwmn0219R: tgcctcgaagcctaatgatattttttttaag [This study]
- pSP-Luc Xhol: acrggcctctgccgactcggatgatagcaaa [This study]
- oBS49: tagttttattagtattattatttta [This study]
- oBS51: attcaatatatttaaaaatttaca [This study]
- oBS53: aggtactactgagtagcactttttt [This study]
- oBS54: aagttctactatattttttttttttagatgatagc [This study]

**primer extension**

- pe_esxA_1: BIOTIN-ccataactagaaacacctttcgg [This study]
- pe_esxA_2: BIOTIN-tgatctcctctgactcctg [This study]
- esxA_term-r: tgcgtatcacatgctatccctttagt [This study]

a Restriction sites are underlined. Capital letters show the att sites.
Protease activity
The proteolytic activity of *S. aureus* strains was determined on skim milk (Becton Dickinson, 75 g l\(^{-1}\)) agar plates as clear zones surrounding colonies.

Hemolytic activity
To compare the hemolytic activity, *S. aureus* strains were grown on sheep blood agar and the clear halos around the colonies were analyzed.

Susceptibility testing
Plates containing an antibiotic gradient were prepared and inoculated by swabbing a 0.5 McFarland cell suspension in physiological NaCl solution along the gradient as described before [27]. Growth was read after 24 h and 48 h of incubation at 35°C. Teicoplanin and oxacillin minimal inhibitory concentrations (MICs) were determined using Etests according to the manufacturer’s instructions (AB-Biodisk, Solna, Sweden).

Results and discussion
Transcriptional analysis of *esxA*
The 294 bp *esxA* gene (*nwmn_0219*, GenBank accession no. NC_009641), coding for a small secreted protein involved in staphylococcal virulence, is the first of at least nine genes of the *ess* gene cluster encoding the type VII-like ESX-1 secretion pathway (Ess) in *S. aureus* (Figure 1A) [14,15]. Although *esxA* seems to belong transcriptionally to the *ess* gene cluster [43], transcriptional profiling produced one single *esxA*-specific transcript with a size of about 0.45 kb appearing in early growth phase after 1 h and increasing slightly within time (Figure 1B). No *esxA*-specific signals were detected in the corresponding Δ*esxA* mutant BS304, confirming the *esxA* deletion. The deletion of *esxA* had no polar effects on the expression of the downstream *ess* genes, nor on the divergently transcribed gene directly upstream of *esxA*, predicted to be involved in staphyloxanthin synthesis [37,44,45] (data not shown). Our results...
suggest that esxA is located on a monocistronic transcript and is not co-transcribed with the remaining genes of the ess gene cluster.

esxA promoter and terminator sequence analysis
In a microarray of strain Newman, esxA transcription was found to be upregulated by the σ^A-controlled yabf-spoVG operon [10]. Searching the nucleotide sequence upstream of the esxA ORF for potential σ^A (TTGACA-16/18-TATAAT) [46,47] and σ^B (GTTTAA-12/15-GGGTAT) [30] consensus promoter sequences and for a ribosomal binding site (AGGAGG) [48], we identified 80 bp upstream of esxA a putative σ^A promoter (TatACA-17-TATTAT), and 155 bp upstream of esxA a potential σ^B promoter (GgTTAA-12-GGGTAT). A proposed ribosomal binding site (RB5, AGGAGG) was located 9 bp upstream of the esxA start codon (Figure 1A). Fourteen bp downstream of the esxA stop codon we identified a putative Rho independent terminator consisting of a 13 bp inverted repeat with a minimal free energy ΔG of -17 kcal/mol as calculated by mfold [49].

Primer extension analysis located the transcriptional start point (TSP) of esxA 74 bp upstream of the start codon of esxA (Figure 1A-C). It was preceded by the predicted -10 and -35 σ^A promoter elements, and further up by the σ^B promoter.

To verify and compare the function of the putative σ^A and σ^B promoter sequences, we cloned the esxA promoter region upstream of the firefly luciferase reporter gene and analyzed the luciferase activity of this construct, pexxAp-luc^*, as well as of constructs containing either a deletion of the σ^A or σ^B promoter (pexxApΔσ^A-luc^*, pexxApΔσ^B-luc^*). Whereas the relative luciferase activities of pexxAp-luc^* and pexxApΔσ^B-luc^* after 3 h of growth were comparable, pexxApΔσ^A-luc^* showed almost no activity, suggesting that esxA possesses a σ^A-dependent promoter (Figure 2). We could rule out a direct involvement of σ^B in the control of the esxA promoter, furthermore, by testing the esxA upstream region in the heterologous two-plasmid system that was established to identify σ^B-dependent S. aureus promoters [30]. The upstream region of esxA was cloned into the reporter plasmid pSB40N resulting in plasmid pexxAp which then was introduced into E. coli DH5α containing either pAC7-sigB, expressing the S. aureus sigB gene from an inducible promoter, or the empty plasmid pAC7. If the S. aureus σ^B - E. coli RNA polymerase core enzyme hybrid recognized the esxA promoter, dark blue colonies would be expected on the indicator LBACX-ARA agar [29] in combination with pAC7-sigB, as with the σ^B-dependent promoters of asp23 or yabf (positive controls); if not, uncolored colonies would be expected, as with the σ^B-independent promoter of capA or the empty pSB40N (negative controls). In contrast, transformants containing the empty pAC7 vector should produce uncolored colonies. However, both combinations, pexxAp with either pAC7 or pAC7-sigB, developed an identical only light blue color in E. coli DH5α, indicating that the esxA promoter was recognized weakly by an E. coli RNA polymerase, but that the observed transcriptional activity was independent from σ^B (data not shown). Overall, the results of the esxA promoter and terminator sequence analyses supported a monocistronic transcription of esxA from a σ^A-dependent promoter.

Effect of σ^A and σ^B-controlled SpoVG on esxA expression
To differentiate between the effect of σ^B and of the σ^A-controlled yabf-spoVG on the transcriptional control of esxA, we followed the luciferase activity of the esxA promoter-reporter fusion in pexxAp-luc^* during the growth cycle in parental strain Newman, the corresponding ΔrsbUVW-sigB mutant (IK184), and in the Δyabf-spoVG mutant (SM148). The luciferase activity increased in the parent Newman in a growth phase dependent manner from the exponential towards the stationary phase and declined thereafter (Figure 3A). The course of luciferase activity in the Δyabf-spoVG mutant SM148 and in the ΔrsbUVW-sigB mutant IK184 was comparable but the overall activity was reduced by a factor of two in SM148, whereas it was two up to four times higher in IK184. These effects were also mirrored by the intensity of the esxA specific transcripts (Figure 3B). Since esxA transcription in strain MS64 [24], a mutant with a stop in sigB inactivating σ^B, was indistinguishable from that

![Figure 2 σ^A-dependence of the esxA promoter](image-url)
in IK184, we could assign the upregulation of \textit{esxA} transcription to the loss of \textit{sigB} and exclude any contributions of \textit{rsbUVW} (data not shown).

To determine if either \textit{yabJ} or \textit{spoVG} inactivation was responsible for the reduction of \textit{esxA} transcription, we complemented Newman, SM148 and IK184 in \textit{trans} with a series of plasmids expressing constitutively either \textit{yabJ} (\textit{pyabJ}), \textit{spoVG} (\textit{pspoVG}), or \textit{yabJ-spoVG} (\textit{pyabJspoVG}), circumventing the requirement of \textit{sigB} to transcribe the \textit{yabJ-spoVG} operon. Northern blot analysis revealed that the constructs containing \textit{spoVG} or \textit{yabJ-spoVG}, but not the one carrying \textit{yabJ}, did restore the \textit{esxA} transcription to wild type level in SM148 (Figure 3C). In IK184, showing stronger \textit{esxA} transcription signals than the wild type, the \textit{esxA} transcription was even further enhanced by the complementation with
spoVG or pyabsSpoVG, confirming that SpoVG, but not YabJ, had a positive effect on esxA expression in presence and absence of $\sigma^B$. However, the fact that esxA transcription was strongly induced in IK184 lacking not only $\sigma^B$, but consequently also the $\sigma^B$-dependent SpoVG, suggested that esxA transcription may be activated by SpoVG but repressed by other $\sigma^B$-dependent factors.

Influence of major regulators SarA, RNAIII and ArlR on esxA

As $\sigma^B$ and SpoVG had opposite effects on esxA expression, we searched for further $\sigma^B$-dependent regulators that might be involved in esxA control, namely the two major regulators of S. aureus, the agr system with its effector molecule RNAIII; and the transcriptional regulator SarA. A further candidate was ArlR, the response regulator of the ArlRS two-component system, reported to be activated by $\sigma^B$ in strain Newman, and promoting together with SpoVG capsule formation [9]. The transcript intensity of esxA in Newman compared to that in its isogenic $\Delta$sarA (LR15), $\Delta$agr (KS186) and $\Delta$arIR (SM99) mutants during growth, revealed a strong upregulation of esxA in LR15, a downregulation in KS186 and an even stronger attenuation in SM99 (Figure 4A), suggesting that SarA acts as repressor, and RNAIII and ArlR as activators of esxA transcription. This was confirmed by the level of luciferase activity of pesxAp-luc$^+$ during growth, which was highly increased in the $\Delta$sarA mutant (BS309), and lower in the $\Delta$agr (BS310) and almost absent in $\Delta$arIR (SM99) mutants compared to the wild type Newman (Figure 4B). Interestingly, as in capsule synthesis, SpoVG and ArlR acted as elements enhancing the esxA expression [9].

Influence of EsxA on regulatory elements and itself

EsxA itself had no influence on the signal intensity or activity of any of the above regulatory genes, neither on asp23, as an indicator of $\sigma^B$ activity [37,44,50], nor on spoVG, arlR, sarA or RNAIII, when comparing their expression in strain Newman and in the $\Delta$esxA mutant BS304 during the growth cycle (Additional file 1). We could also rule out any autoregulatory effects of EsxA on its own transcription, since luciferase activity patterns of pesxAp-luc$^+$ were congruent over the entire growth cycle in Newman and BS304 (data not shown).

Figure 4 Effect of SarA, agr and ArlR on esxA expression. A. Northern blot of esxA in Newman, and the $\Delta$sarA (LR15), $\Delta$agr (KS186) and $\Delta$arIR (SM99) mutants over growth. The ethidium bromide-stained 16S rRNA pattern is shown as an indication of RNA loading. B. Transcriptional activity of the esxA promoter in strain Newman (squares), $\Delta$sarA mutant BS309 (stars/dots), $\Delta$agr mutant BS310 (triangles), and $\Delta$arIR mutant SM99 (diamonds). Growth was followed by measuring the OD$_{600}$ (open signs), and the activity of the esxA promoter-reporter construct was determined by the luciferase activity of pesxAp-luc$^+$ (filled signs). The strains BS309 and BS310 are isogenic to LR15 and KS186, respectively, except for an exchanged resistance marker in the inactivated loci allowing the selection and maintenance of pesxAp-luc$^+$. 

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Influence of SarA, RNAIII, σ^B, ArlR and SpoVG on each other

An overview of the regulatory network influencing esxA transcription is given in Figure 5, including also the mutual interactions of the different regulators: σ^B activity was found to be comparable in all strains tested, excluding secondary effects on esxA transcription due to an altered σ^B activity (Additional file 2). We confirmed the previously reported positive influence of σ^B on arlRS and yah/spoVG transcription [7,9], as well as on sra transcription [3,7]. In contrast, we could not detect any major changes in RNAIII transcript intensity in σ^B mutants, although some studies suggest that σ^B activity is reducing the RNAIII level [3,4] (Additional file 2).

Further, minor changes in transcription were observed in the ΔsarA mutant where RNAIII was downregulated and arlR transcripts were slightly upregulated, and in the ΔarlR mutant where sarA transcription was increased (Additional file 2: Figure S2A). However, these dependencies could not explain the changes in esxA transcription in the corresponding mutants.

Phenotypic characteristics of the ΔesxA mutant

The successful deletion of esxA reported here, and the superimposable growth rates of wild type and esxA mutant in complex LB medium, confirmed that EsxA was not essential for growth in vitro (data not shown). The growth defects observed in sigB and arlR mutants, the former affecting late [37] and the latter reducing early growth stages [19], can therefore also not depend on altered EsxA expression. Although σ^B and SpoVG are known to influence extracellular proteolytic activities [9], and σ^B is known to repress hemolytic activity in S. aureus [4,7,37], EsxA did neither affect proteolytic nor hemolytic activities in BS304 (data not shown). As the activity of the sigma factor σ^B and the σ^B-controlled SpoVG positively influences methicillin and glycopeptide resistance in methicillin resistant S. aureus (MRSA) and in glycopeptide intermediate resistant S. aureus (GISA) [8,51-55], we deleted esxA in MRSA strain BB1002 [26] and GISA strain NM143 [27]. However, resistance levels of the ΔesxA mutants BS307 and BS308 to oxacillin and teicoplanin, respectively, were identical to those of the parent strains, when measured by Etest (Table 3), as well as by antibiotic gradient plates, which allow the detection of very small differences in resistance (data not shown). These results suggest that EsxA, which enhances abscess formation in mice and is thought to act either as transport chaperone or adaptor protein [18], primarily plays a role as extracellular virulence factor in pathogenesis.

Conclusion

Our data suggest that the repression of esxA by σ^B is due the σ^B-induced transcription of sarA, leading to a strong and dominating SarA-mediated repression of esxA. The activation of esxA transcription, on the other hand, is stimulated by the agr quorum sensing system,
the response regulator ArlR, and the effector protein SpoVG; whereby arlR is controlled indirectly, and spoVG directly by σB. Thereby the activating effect of ArlR seems to be more profound than the effect of SpoVG and agr. Moreover, virulence gene regulation in *S. aureus* is very complex and additional factors might contribute to the regulation of *exsA* transcription.

The mode of function of SpoVG, named after the stage V sporulation protein G in *Bacillus subtilis* [7], and SpoVG homologues in other bacterial species is yet unknown, nor have any SpoVG interacting partners been reported. SpoVG does not affect σB activity as seen from the expression of *asp23*, which is a measure of σB activity in *S. aureus*. SpoVG does also not interfere with the transcription of *sarA, arlRS* nor *agr* in strain Newman.

By which mechanisms SpoVG counteracts the postulated SarA-mediated repression of *exsA* remains open. The affinity of SarA binding to DNA can be enhanced by phosphorylation [56], but a postulated interaction of SpoVG with SarA or other proteins has yet to be investigated. Interestingly, the same stimulating effect by ArlRS and SpoVG is seen in *S. aureus* capsule synthesis [9]. We therefore can not rule out that SpoVG and ArlR may interact or have some common target. SpoVG by itself seems also to enhance transcription of *exsA* when artificially overexpressed in a *sigB* mutant. The absence of predicted DNA binding motifs in SpoVG may not fully exclude its interaction with nucleic acids or with factors involved in transcription. In conclusion, we have presented here SpoVG, an interesting new player in the regulatory cascade modulating *S. aureus* virulence factors.

**Additional material**

*Table 3 Oxacillin and teicoplanin MICs*  

| Strain   | MIC (µg ml⁻¹) | Oxacillin | Teicoplanin |
|----------|---------------|-----------|-------------|
| Newman  | 0.19          | 4         |             |
| BS304   | 0.19          | 4         |             |
| BB1002  | > 256         | 3         |             |
| BS307   | > 256         | 3         |             |
| NM143   | 0.25          | 12        |             |
| BS308   | 0.25          | 12        |             |

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**Author details**

1. Institute of Medical Microbiology, University of Zurich, Gloriastrasse 32, 8006 Zurich, Switzerland. 2. Cellular and Molecular Microbiology Division, Interfaculty Institute of Microbiology and Infectious Medicine, University of Tübingen, Elfriede-Aulhorn-Strasse 6, 72076 Tübingen, Germany.

**Authors’ contributions**

BS carried out most of the experiments, participated in the design of the study and drafted the manuscript. DAB participated in the transcriptional analysis. BBB conceived the study, and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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