JOURNAL OF BACTERIOLOGY, Sept. 2001, p. 5171–5179 Vol. 183, No. 17
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.17.5171–5179.2001


# Influence of a Functional *sigB* Operon on the Global Regulators *sar* and *agr* in *Staphylococcus aureus*

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Received 24 January 2001/Accepted 18 June 2001

**The growth phase-dependent activity profile of the alternate transcription factor $\sigma^B$ and its effects on the expression of *sar* and *agr* were examined in three different *Staphylococcus aureus* strains by Northern blot analyses and by the use of reporter gene fusion experiments. Significant $\sigma^B$ activity was detectable only in the clinical isolates MSSA1112 and Newman, carrying the wild-type *rsbU* allele, but not in the NCTC8325 derivative BB255, which is defective in *rsbU*. $\sigma^B$ activity peaked in the late exponential phase and diminished towards the stationary phase when bacteria were grown in Luria-Bertani medium. Transcriptional analysis and a *sarP1-sarP2-sarP3* (*sarP1-P2-P3*)-driven firefly luciferase (*luc*+) reporter gene fusion demonstrated a strong $\sigma^B$ activity- and growth phase-dependent increase in *sar* expression that was totally absent in either *rsbU* or Δ*rsbUVWsigB* mutants. In contrast, expression of the *agr* locus, as measured by RNAIII levels and by an *hldp*::*luc*+ fusion, was found to be higher in the absence of $\sigma^B$ activity, such as in *rsbU* or Δ*rsbUVWsigB* mutants, than in wild-type strains. Overexpression of $\sigma^B$ in BB255 derivatives resulted in a clear increase in *sarP1-P2-P3*::*luc*+ expression as well as a strong decrease in *hldp*::*luc*+ expression. The data presented here suggest that $\sigma^B$ increases *sar* expression while simultaneously reducing the RNAIII level in a growth phase-dependent manner.**

*Staphylococcus aureus* is a major human pathogen causing a variety of infections, ranging from minor skin and wound infections to life-threatening diseases (42). Pathogenicity in *S. aureus* is based on a wide range of cell wall-associated and extracellular proteins that are regulated in a coordinate and growth phase-dependent manner. These virulence determinants are controlled among others by the accessory gene regulator *agr* and the staphylococcal accessory regulator *sar* (48). Mutations in either *agr* or *sar* result in mutants that are strongly attenuated in virulence compared to their corresponding parental strains (1, 8, 15, 31).

The *agr* locus regulates the expression of cell wall-associated proteins and secreted exoproteins in response to the density of the bacterial population (37). The proposed function of this regulatory system is to enhance the production of wall-associated adhesins, which interact with the host's matrix proteins, and potential defense factors (protein A) in the early stages of infection. This is followed by the expression of excreted invasion factors, such as hemolysins, proteases, and lipases, that are suggested to be involved in the dissemination of the organism from the primary site of infection once the infection has been established (58). The *agr* locus comprises two divergent transcriptional units, RNAII and RNAIII, which are transcribed from the *agrP2* and *agrP3* promoters, respectively (Fig. 1B) (reviewed in reference 46). RNAII encodes a four-gene operon, including a two-component signal transduction system that responds to the concentration of a secreted and processed peptide pheromone, which is encoded within the operon itself. The primary function of the RNAII gene products is to activate the *agrP2* and *agrP3* promoters, significantly aided by SarA. Transcription from the *agrP3* promoter results in a 510-nucleotide RNA molecule (RNAIII), which appears to be the effector molecule of the positive and negative regulation of virulence genes that are controlled by the *agr* locus (36, 49). RNAIII is thought to regulate most target genes at the level of transcription but has also been shown to influence the translation of some genes (45, 49) and contains a small open reading frame coding for delta hemolysin (*hld*).

SarA, the major functional protein encoded by the *sar* locus, is generally believed to be required for the activation of expression of the *agr* locus (20, 21, 46) and influences the regulation of several virulence factors independently from *agr* (7, 11, 14, 19, 41, 60). SarA is essentially involved in the capacity of *S. aureus* to survive inside of polymorphonuclear neutrophils (33), and the ability of *S. aureus* to enter mammalian cells and induce apoptosis is supposed to be dependent on factors regulated by *sar* and *agr* (58). SarA expression itself is controlled by three different, tandemly arranged promoters (Fig. 1A) in a growth phase-dependent manner (3, 23, 43). Although one of these transcripts, *sarC*, was shown to be controlled by the alternative transcription factor $\sigma^B$ in vitro (10, 28, 43), the inadvertant use of an *rsbU* mutant to compare *sar* expression in a *sigB* mutant may have wrongly suggested that $\sigma^B$ was involved neither in the transcriptional control of the *sar* locus nor in *agr* expression (12, 14, 17). The strains used in those studies were recently shown to possess almost no $\sigma^B$ activity due to a mutation in the *rsbU* gene, which encodes a positive regulator of $\sigma^B$ (30). The transcription factor $\sigma^B$ itself, organized in the *rsbUVWsigB* operon, is supposed to be activated by a cascade encompassing RsbU, an RsbV-specific phosphatase, the anti-anti-sigma factor RsbV, and the anti-sigma factor RsbW.

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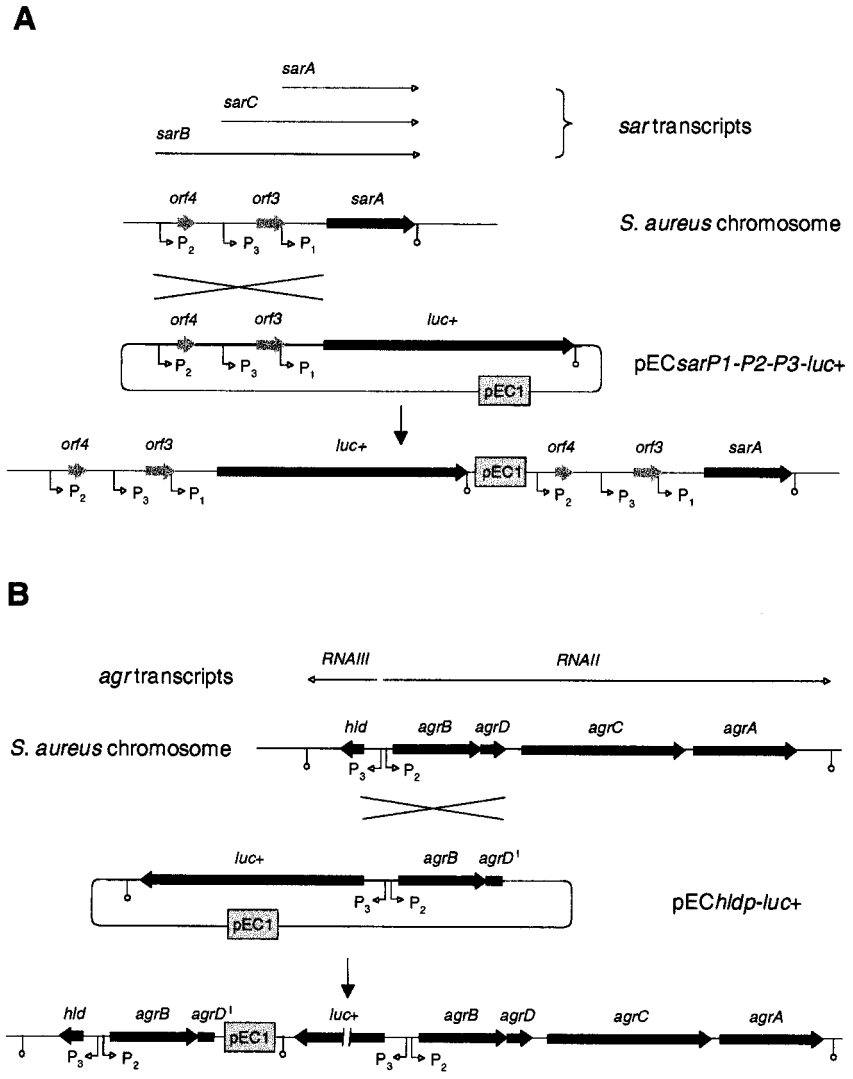


FIG. 1. Genetic organization of the *sar* and *agr* loci of *S. aureus*. Genetic organization of the *sar* locus (A) and the *agr* locus (B) of *S. aureus* and schematic representation of the integration of *sarP1-P2-P3*::*luc*+ or *hldp*::*luc*+ fusion constructs into the *S. aureus* chromosome by single crossover. For a description of construction of the plasmids pEC*sarP1-P2-P3-luc*+ and pEC*hldp-luc*+ and integration of the constructs into the *S. aureus* chromosome, see Materials and Methods. Open reading frames, promoters, and respective transcripts are indicated.

In this study we demonstrate by the use of transcriptional analyses and reporter gene fusion experiments in three different genetic backgrounds that transcription of both the *sar* and *agr* loci are clearly influenced by the $\sigma^B$ activity in *S. aureus* strains harboring a functional *sigB* operon.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* was routinely grown in Luria-Bertani (LB) medium at 37°C and at 200 rpm. Antibiotics were used at the following concentrations: for erythromycin and tetracycline, 10 μg ml$^{-1}$; for ampicillin, 50 μg ml$^{-1}$.

**General methods.** All DNA manipulations, basic molecular methods, and handling of *Escherichia coli* were performed in accordance with standard protocols (54). Genetic manipulation of *S. aureus* was done as described earlier (39). The general transducing phage 80α was used for transductions.

**Construction of pEC*sarP1-P2-P3-luc*+ and pEC*hldp-luc*+.** A DNA fragment covering 867 bp of the *sar* promoter region of *S. aureus* RN4220 was generated by PCR using an upstream primer (5′-CGGTACC*GTTGATTTGGGTAGTATG C-3′) including a Kpn*I linker (underlined) and a downstream primer (5′-TTG*C CATGGTTAAAACCTCCC-3′) including a Nco*I site (underlined), with italic nucleotides corresponding to positions 5 to 24 and 852 to 872 of the sequence found under GenBank accession no. U46541, respectively. For *hldp*::*luc*+, a DNA fragment covering 1 kb of the *agr* locus of *S. aureus* RN4220 was generated by PCR using an upstream primer (5′-*GTGCCATGGAAATCACTCCTTCC*-3′) including a *Nco*I site (underlined) and a downstream primer (5′-*TGGTACCTC AACTTCATCCATTATG*-3′) including a *Kpn*I site (underlined), with italic nucleotides corresponding to positions 397 to 419 and 1348 to 1372 of the sequence found under GenBank accession no. AF230358, respectively. The PCR products obtained were digested with *Kpn*I and *Nco*I and cloned in frame with the 5′ end of the luciferase gene of plasmid pSP-*luc*+. Sequence analysis and comparison confirmed the identity of the constructs to the RN6390 sequence or RN4220 sequence, respectively. A 2.5-kb *Kpn*I-*Eco*RI fragment, including the *sar* promoter region fused to the luciferase coding region, or a 2.6-kb *Kpn*I-*Eco*RI fragment, including the *hld* promoter region fused to the luciferase coding region, was subsequently cloned into the suicide plasmid pEC1 (9) to obtain the plasmids pEC*sarP1-P2-P3-luc*+ (Fig. 1A) and pEC*hldp-luc*+ (Fig. 1B), respectively. The plasmids obtained were transformed by electroporation into RN4220 and transduced into different *S. aureus* genetic backgrounds.

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or phenotype[a] | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH10B | F$^-$ ϕ80d*lac*ZΔM15 *recA1* | Gibco |
| *S. aureus* | | |
| RN4220 | NCTC8325-4, r$^-$ m$^+$ (restriction minus, modification plus), *rsbU* | 38 |
| BB255 | Essentially the same as NCTC8325, *rsbU* | 4 |
| Newman | ATCC25904; clinical isolate, high level of clumping factor, *rsbU*$^+$ | 24 |
| MSSA1112 | Clinical isolate, *bla rsbU*$^+$ | 25 |
| GP266 | RN4220, *rsbU*$^+$ *sigB1*(Am) Tc$^r$ | 6 |
| GP268 | BB255, *rsbU*$^+$*V*$^+$ *W*$^+$*sigB*$^+$ Tc$^r$ | 30 |
| IK181 | BB255, Δ*rsbUVWsigB*::*erm*(B) Em$^r$ | 40 |
| IK184 | Newman, Δ*rsbUVWsigB*::*erm*(B) Em$^r$ | 40 |
| MB32 | Newman, *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Em$^r$ | 30 |
| MB33 | BB255, *rsbU asp23*$^+$*asp23p*::pEC*asp23p-luc*+ Em$^r$ | 5 |
| MB39 | MSSA1112, Δ*rsbUVWsigB*::*erm*(B) Em$^r$ | This study |
| MB49 | GP268, *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Tc$^r$ Em$^r$ | 30 |
| MB69 | Newman, Δ*rsbUVWsigB*::*erm*(B) *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Tc$^r$ Em$^r$ | 30 |
| MB70 | MSSA1112, Δ*rsbUVWsigB*::*erm*(B)*asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Tc$^r$ Em$^r$ | This study |
| MB73 | MSSA1112, *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Em$^r$ | This study |
| MB90 | BB255, Δ*rsbUVWsigB*::*erm*(B) *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Tc$^r$ Em$^r$ | 30 |
| MB94 | BB255, *rsbU*$^+$ *hld*$^+$ *hldP*::*luc*+ Tc$^r$ Em$^r$ | This study |
| MB95 | BB255, *rsbU hld*$^+$ *hldp*::pEC*hldp-luc*+ Em$^r$ | This study |
| MB97 | Newman, *hld*$^+$ *hldp*::pEC*hldp-luc*+ Em$^r$ | This study |
| MB98 | BB255, *rsbU sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Em$^r$ | This study |
| MB100 | Newman, *sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Em$^r$ | This study |
| MB101 | Newman, *sigB1*(Am) *sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Tc$^r$ Em$^r$ | This study |
| MB102 | BB255, *rsbU*$^+$ *sigB1*(Am) *sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Tc$^r$ Em$^r$ | This study |
| MB103 | Newman, *sigB1*(Am) *hld*$^+$ *hldp*::pEC*hldp-luc*+ Tc$^r$ Em$^r$ | This study |
| MB104 | MSSA1112, *hld*$^+$ *hldp*::pEC*hldp-luc*+ Em$^r$ | This study |
| MB105 | MSSA1112, *sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Em$^r$ | This study |
| MB112 | MSSA1112, *sigB1*(Am) *hld*$^+$ *hldp*::pEC*hldp-luc*+ Tc$^r$ Em$^r$ | This study |
| MB113 | MSSA1112, *sigB1*(Am) *sar*$^+$ *sar-P1-P2-P3*::pEC*sar-P1-P2-P3-luc*+ Tc$^r$ Em$^r$ | This study |
| Plasmids | | |
| pSP-*luc*+ | Ap$^r$, firefly luciferase casette vector | Promega |
| pEC1 | Ap$^r$ Em$^r$, 1.45-kb *Cla*I *erm*(B) fragment of Tn*551* in pUC18 | 9 |
| pTX15 | Tc$^r$ $P_{xyl}$, staphylococcal origin of replication | 50 |
| pIK64 | Tc$^r$, pTX15 derivative, containing *sigB* under the control of the xylose-inducible promoter $P_{xyl}$ | 40 |
| pSP*sarP1-P2-P3* | Ap$^r$, 867-bp PCR fragment of *sar* promoter from strain RN4220 in pSP-*luc*+ | This study |
| pSP*hldp* | Ap$^r$, 1-kb PCR fragment of *hld* promoter from strain RN4220 in pSP-*luc*+ | This study |
| pEC*sarP1-P2-P3-luc*+ | Em$^r$, 2.5-kb *Kpn*I-*Eco*RI *sarP1-P2-P3-luc*$^+$ fragment of pSP*sarP1-P2-P3* in pECI, *S. aureus* integration vector that inserts into the *sar* promoters (*sarP1-P2-P3*) | This study |
| pEC*hldP*::*luc*+ | Em$^r$, 2.6-kb *Kpn*I-*Eco*RI *hldp-luc*+ fragment of pSP*hldP* in pEC1, *S. aureus* integration vector that inserts into the *hld* promoter (*hldp*) | This study |

[a] Abbreviations are as follows: Ap$^r$, ampicillin resistant; Em$^r$, erythromycin resistant; Tc$^r$, tetracycline resistant.

**Northern blot analyses.** Isolation of total RNA was done as described by Cheung et al. (16). Eight micrograms of total RNA of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid running buffer (20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA [pH 7]). Blotting of RNA onto a positively charged nylon membrane (Roche, Basel, Switzerland) was performed with a vacuum blotter (Pharmacia, Uppsala, Sweden). The intensities of the 23S and 16S rRNA bands stained with ethidium bromide were verified to be equivalent in all the samples before transfer. Labeling and hybridization were done by the use of the digoxigenin labeling and detection kits according to the manufacturer's instructions (Roche). The following specific primers were used to generate the digoxigenin-labeled DNA probes by PCR labeling: Sa*sarA*+, 5′-AGGGAGGT TTTAAACATGGC-3′; Sa*sarA*−, 5′-CTCGACTCAATAATGATTCG-3′ (nucleotides 851 to 870 and 1177 to 1196 of the sequence found under GenBank accession no. U46541); *RNAIII*+, 5′-GTGATGGAAAATAGTTGATGAG-3′; *RNAIII*−, 5′-GTGAATTTGTTCACTGTGTCG-3′ (nucleotides 453 to 474 and 333 to 353 of the sequence under GenBank accession no. AF230358).

**Luciferase assay.** Bacterial cells from overnight cultures containing the appropriate antibiotic were diluted with fresh LB medium to an optical density at 600 nm ($OD_{600}$) of 0.01. Freshly diluted cells were incubated without antibiotics at 37°C and at 200 rpm. *S. aureus* cells, obtained at different growth stages, were harvested by centrifugation at 11,000 × *g* during 1 min at room temperature, and the cell pellets were resuspended in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$ [pH 7.3]) to an $OD_{600}$ of 10. Luciferase activity was that determined by rapidly mixing PBS-resuspended cells (10 μl) with an equal volume of Luciferase Assay Substrate (Promega, Madison, Wis.). Luminescence was measured on a Turner Designs TD-20/20 Luminometer (Promega) for a time period of 10 s with a delay of 2 s.

**Fibronectin binding assay.** Binding of *S. aureus* to fibronectin was measured quantitatively in microtiter plates, by a slight modification of a previously described method (44). Briefly, 50 μl of human fibronectin (Sigma, Buchs, Switzerland) was serially diluted twofold from a starting concentration of 500 μg/ml in PBS. Bacterial cells grown to exponential or stationary phase were harvested by centrifugation and washed in PBS, and 20 μl of a suspension ($5 \times 10^8$ CFU)

was added to the fibronectin dilutions. The lowest concentration of fibronectin triggering clumping after overnight incubation at 4°C was recorded as the titer.

## RESULTS

**$\sigma^B$ activity in *S. aureus*.** The $\sigma^B$ activities of the two genetically distinct strains Newman and MSSA1112 and their respective Δ*rsbUVWsigB* mutants, as well as those of the *rsbU* strain BB255 and its derivative GP268, transformed with the *rsbU* wild-type allele, were analyzed during growth by the use of the *asp23* reporter gene system (30). The two clinical isolates MSSA1112 and Newman possessed quite similar $\sigma^B$ activity profiles in LB medium, with a maximal $\sigma^B$ activity in late exponential growth phase followed by a significant decrease thereafter (Fig. 2B and C). The $\sigma^B$ activity profile obtained for strain GP268 (MB49) was comparable to those found for strains MSSA1112 (MB73) and Newman (MB32), while its parental strain produced almost no $\sigma^B$ activity throughout the whole growth cycle (Fig. 2A). Additionally, all three Δ*rsbUVWsigB* mutants were unable to produce any $\sigma^B$-dependent activity at all. The unexpected strong decrease in $\sigma^B$ activity that was observed from the onset of stationary phase was confirmed by monitoring the transcription of the $\sigma^B$-dependent genes *asp23* and *sigB* in Northern blot analyses. Both transcripts were found to be the most abundant during late exponential growth phase, and their levels were drastically reduced during stationary phase (data not shown).

**Influence of $\sigma^B$ on the expression of the *sar* locus.** Northern blot analyses with the *sarA* gene as a probe showed strong *sarA* transcription, originating from the $\sigma^A$-dependent *sarP1* promoter, during exponential growth ($OD_{600}$, 0.3 to 1.5) and declining with the onset of stationary phase in all strains analyzed (Fig. 3). A similar time course but much weaker transcription was observed in all strains for *sarB*, originating from the $\sigma^A$-dependent *sarP2* promoter. In contrast, $\sigma^B$-dependent transcription of *sarC* from the *sarP3* promoter was detectable only in the wild-type strains MSSA1112 and Newman, not in BB255 or any of the Δ*rsbUVWsigB* mutants. *sarC*-specific transcripts were detectable abundantly from late exponential growth phase up to stationary phase ($OD_{600}$, 1.5 to 5.0). Reporter gene fusion experiments with the luciferase gene *luc*+ fused to the *sarP1, sarP2*, and *sarP3* (*sarP1-P2-P3*) promoters suggested an increased SarA production in strains MSSA1112 and Newman with the beginning of late exponential growth phase that paralleled the time course of the overall *sar* transcripts observed in those strains. No such increase in luciferase activity was detectable with the *rsbU* mutant BB255 (Fig. 4A) or in any of the Δ*rsbUVWsigB* mutants. Plasmid pIK64 ($P_{xyl}$::*sigB*)-mediated xylose-induced overexpression of $\sigma^B$ in the BB255 derivative MB98 resulted in a strong increase in *sarP1-P2-P3*::*luc*+ expression (Fig. 5A), which was not observed with the control plasmid pTX15 (Fig. 5B), unambiguously proving $\sigma^B$ to influence *sar* expression directly or indirectly.

**Influence of $\sigma^B$ on the expression of the *agr* locus.** Comparison of the growth phase-dependent transcription of RNAIII in Northern blot analyses revealed low transcription levels and a delayed induction of RNAIII expression in the wild-type strains MSSA1112 and Newman compared to results for their corresponding Δ*rsbUVWsigB* mutants (Fig. 6B and C). The RNAIII expression profile of strain GP268 (BB255 $rsbU^+$) paralleled the transcription profiles found for strains MSSA1112 and Newman, with expression being significantly delayed and at a lower level than that for BB255 (Fig. 6A). Reporter gene fusion experiments, using the luciferase gene *luc*+ fused to the *hld* gene, carried by RNAIII (Fig. 1B), confirmed these data. The RNAIII-representing luciferase activity profiles of strains carrying the wild-type *rsbU* allele were significantly lower than those for Δ*rsbUVWsigB* mutants and

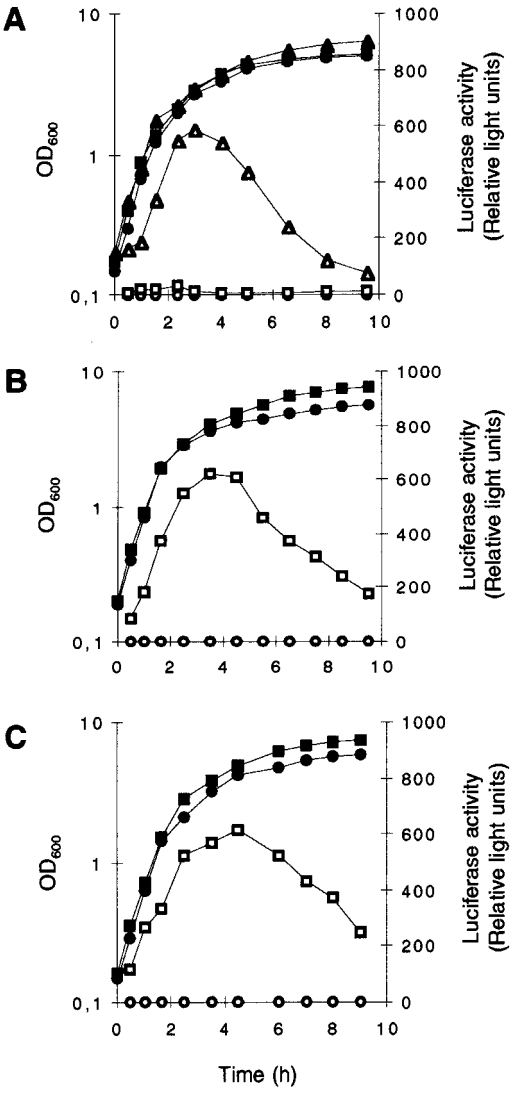


FIG. 2. $\sigma^B$ activity during growth of *S. aureus*. Expression of *asp23*::*luc*+ during growth of *S. aureus* strains BB255 (A), MSSA1112 (B), Newman (C), and their respective *sigB* mutants. Strains were grown in LB medium at 37°C. Bacterial growth was measured as the $OD_{600}$ (solid symbols). $\sigma^B$ transcriptional activity was determined by measuring the luciferase activity of Luc+ (open symbols), the product of the *luc*+ reporter gene fused to the $\sigma^B$-dependent promoters of *asp23* (*asp23p*). (A) Squares, *S. aureus* strain MB33 (BB255, *asp23p*::*luc*+); triangles, strain MB49 (BB255, *rsbU*+ *asp23p*::*luc*+); circles, strain MB90 (BB255, Δ*rsbUVWsigB asp23p*::*luc*+). (B) Squares, *S. aureus* strain MB73 (MSSA1112, *asp23p*::*luc*+); circles, strain MB70 (MSSA1112, Δ*rsbUVWsigB asp23p*::*luc*+). (C) Squares, *S. aureus* strain MB32 (Newman, *asp23p*::*luc*+); circles, strain MB69 (Newman, Δ*rsbUVWsigB asp23p*::*luc*+).

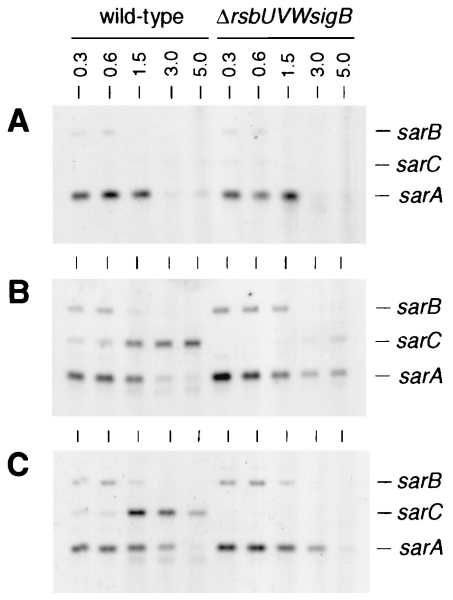


FIG. 3. Northern blot analyses of the *sar* locus. Total RNAs (8 μg/lane) of *S. aureus* strains BB255 (A), MSSA1112 (B), Newman (C), and their respective *sigB* mutants were blotted onto positively charged nylon membranes and subjected to Northern blot analyses. RNAs were obtained from cells grown in LB medium at 37°C and harvested at different growth stages (indicated as $OD_{600}$ values [numbers above the lanes]). The blotted membranes were hybridized using a digoxigenin-labeled DNA probe specific for *sarA* (for details on the construction, see Materials and Methods). The RNA molecular weight marker I (Roche) was used as a size marker. Relevant transcript signals are indicated.

BB255, respectively (Fig. 7). Overexpression of $\sigma^B$ in the BB255 derivative MB95, harboring plasmid pIK64, resulted in a strong decrease in *hldp*::*luc*+ expression from that of the control (Fig. 8), corroborating the negative regulatory effect of $\sigma^B$ on *agr* expression.

**Fibronectin binding activity.** Since *sar* and *agr* are known to influence the ability of *S. aureus* to bind to fibronectin (55, 60), the effects of RsbU on the fibronectin binding capacity were determined with strain BB255 and its derivatives (Table 2). Strain GP268, carrying an intact *sigB* operon, showed a more than 100-fold-lower fibronectin-clumping titer than its *rsbU*-defective parent, BB255, irrespective of the growth phase, while no difference was apparent between BB255 and its Δ*rsbUVWsigB* mutant.

## DISCUSSION

Most of our knowledge of the regulation of $\sigma^B$ in *S. aureus* has been adapted from the well-characterized $\sigma^B$ regulon of the closely related soil bacterium *Bacillus subtilis* (reviewed in reference 34). In this organism, $\sigma^B$ has been shown to function as a stress- and stationary phase-specific transcription factor. In *B. subtilis*, activation of $\sigma^B$ appears to be basically dependent on the activity of the two RsbV-specific phosphatases, RsbU and RsbP, with the latter being essential for the stationary phase and energy stress-dependent activation of $\sigma^B$ (56). RsbU was found to be of importance only for the environmental stress activation of this sigma factor (57, 59, 61). The data

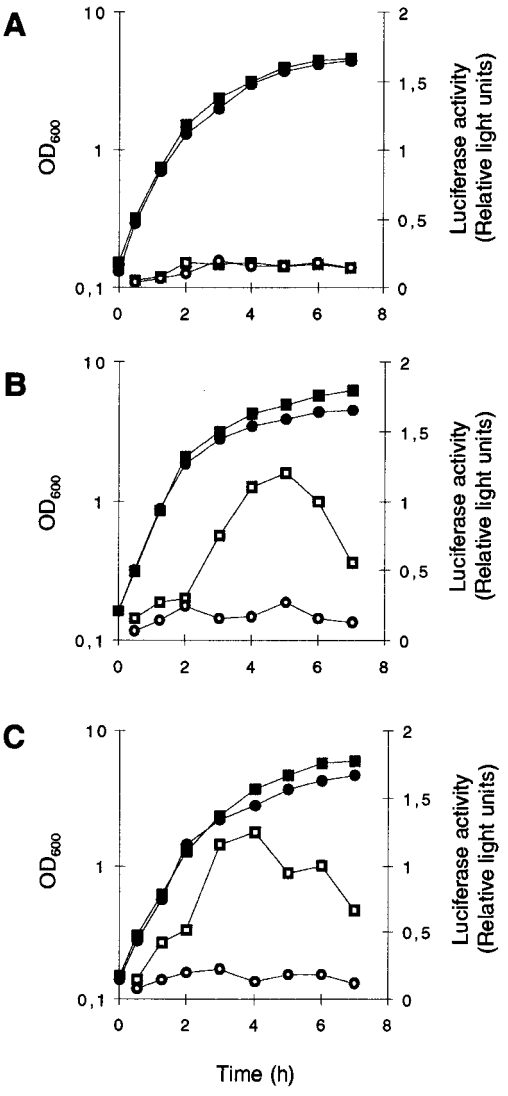


FIG. 4. Role of $\sigma^B$ in the regulation of *sarP1-P2-P3*::*luc*+ expression during growth. *S. aureus* derivatives of strains BB255 (A), MSSA 1112 (B), Newman (C), and their respective *sigB* mutants were grown in LB medium at 37°C. Bacterial growth was measured by $OD_{600}$ (closed symbols). *sarP1-P2-P3*::*luc*+ expression was determined by measuring the luciferase activity of the reporter gene *luc*+ (open symbols). (A) Squares, *S. aureus* strain MB98 (BB255, *sarP1-P2-P3*::*luc*+); circles, strain MB102 (BB255, *sigB sarP1-P2-P3*::*luc*+). (B) Squares, *S. aureus* strain MB105 (MSSA1112, *sarP1-P2-P3*::*luc*+); circles, strain MB113 (MSSA1112, *sigB sarP1-P2-P3*::*luc*+). (C) Squares, *S. aureus* strain MB100 (Newman, *sarP1-P2-P3*::*luc*+); circles, strain MB101 (Newman, *sigB sarP1-P2-P3*::*luc*+).

presented here and elsewhere (6, 30) instead suggest $\sigma^B$ of *S. aureus* to be a transcription factor with the main activity in late exponential growth phase rather than in stationary phase. Additionally, the data clearly demonstrated that the natural *rsbU* mutant BB255 was almost completely unable to express $\sigma^B$ activity, illustrating the importance of RsbU for the overall activity level of $\sigma^B$ in *S. aureus*. Accidentally, nearly all studies of the influence of $\sigma^B$ on the expression of *sar* and *agr* have been carried out with NCTC8325 isogenic backgrounds, harboring the mutation in *rsbU* (12, 14, 23, 43). In consequence,

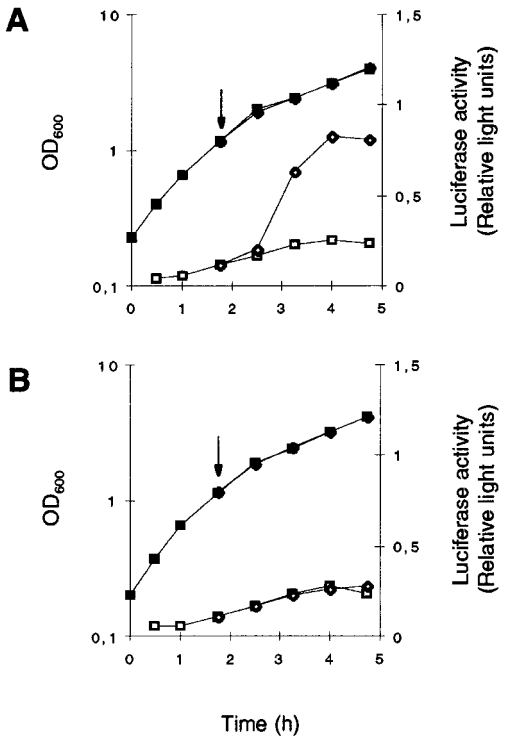


FIG. 5. Effect of overexpressed $\sigma^B$ on the expression of *sarP1-P2-P3*::*luc*+. *S. aureus* derivatives of strain MB98 (BB255, *sarP1-P2-P3*::*luc*+), harboring plasmid pIK64 ($P_{xyl}$::*sigB*) (A) or control plasmid pTX15 ($P_{xyl}$) (B) were grown in LB medium at 37°C. Growth was measured as the $OD_{600}$ (closed symbols). Growing cultures were split into equal parts at the $OD_{600}$ of 1, and overexpression of $\sigma^B$ was induced in one of the parts by supplementing 0.5% xylose (diamonds), while the control part was left without addition (squares). The arrow denotes the time point of supplementation. *sarP1-P2-P3*::*luc*+ expression (open symbols) was determined by measuring the luciferase activity of the reporter gene *luc*+ as described in Materials and Methods.

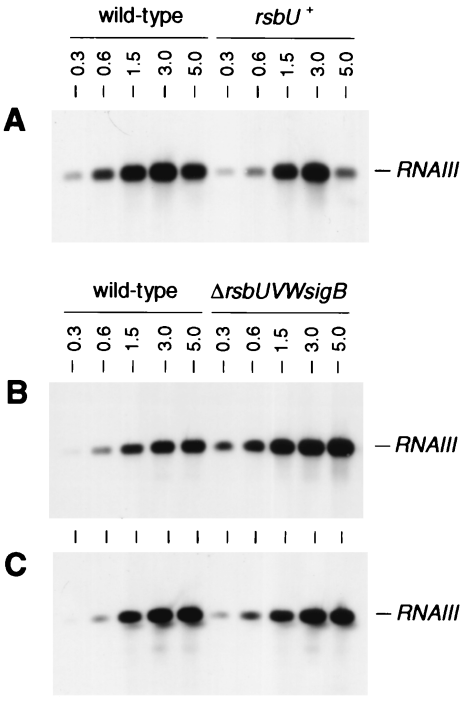


FIG. 6. Northern blot analyses of RNAIII. Total RNAs (8 μg/lane) of *S. aureus* strains BB255 and GP268 (BB255, *rsbU*+) (A), MSSA1112 and MB39 (MSSA1112, Δ*rsbUVWsigB*) (B), and Newman and IK 184 (Newman, Δ*rsbUVWsigB*) (C) were blotted onto positively charged nylon membranes and subjected to Northern blot analyses. RNAs were obtained from cells grown in LB medium at 37°C and harvested at different growth stages (indicated as $OD_{600}$ values above the lanes). The blotted membranes were hybridized using a digoxigenin-labeled DNA probe specific for RNAIII (for details on construction, see Materials and Methods). The RNA molecular weight marker I (Roche) was used as the size marker. Relevant transcript signals are indicated.

the findings presented here call into question the $\sigma^B$-dependent results obtained from such strains.

Transcriptional data and reporter gene fusions of the *sar* locus revealed a strong $\sigma^B$-dependent transcription of *sarC* in *S. aureus* strains harboring an intact *sigB* operon, while no such transcription was detectable in the *rsbU*-deficient strain BB255 nor in any of the Δ*rsbUVWsigB* mutants (Fig. 3 and 4). This results are in contrast to the findings of Bayer et al. (3) and Manna et al. (43), who detected significant amounts of *sarC* transcripts during late exponential phase in the closely related *rsbU*-deficient strain RN6390. It is noteworthy that the reporter gene fusion experiments of Manna et al. (43) performed with strain RN6390 revealed only little activity for the *sarP3* promoter, the level of which was approximately 50-fold lower than that for the *sarP1* promoter. Interestingly, the maximum activity obtained in this study for the *sarP3* promoter was comparable to that found for the *sarP2* promoter. A comparison of the reporter gene data obtained from Manna et al. (43) with the intensities of the different *sar* transcripts presented here in Fig. 3 led us to the conclusion that the RN6390 strain used by Manna et al. also possessed almost no $\sigma^B$ activity. This conclusion is further strengthened by the findings of Cheung et al. (17), who reported neither in vitro nor in vivo activity of the *sarP3* promoter in strain RN6390, in accordance with our finding that the luciferase activity profile of BB255 was indistinguishable from that of its Δ*rsbUVWsigB* mutant. Both findings fit well with our deduction that normal levels of $\sigma^B$-dependent transcription of *sarC* occur only in the presence of a functional RsbU phosphatase. Our data demonstrate that expression of the *sar* locus is significantly upregulated by $\sigma^B$ in a growth phase-dependent manner in *S. aureus* strains harboring an intact *sigB* operon. Thus, we postulate that $\sigma^B$ positively contributes to the overall level of SarA in *S. aureus*. This hypothesis is strengthened by recent findings of Gertz et al. (29), who reported significantly lower SarA levels in a Δ*rsbUVWsigB* mutant of the *rsbU*+ strain COL.

The influence of SarA on the expression of the *agr* locus has been the topic of several studies (13, 20, 21, 35, 46, 53). Even though a factor(s) other than SarA (e.g., ORF3, RAP, and RIP) is suggested to participate in controlling *agr*-related transcription (2, 13, 20, 21), SarA is currently believed to stimulate the expression from both the *agrP2* and *agrP3* promoters, leading ultimately to the upregulation of RNAIII (21; for a review, see reference 48). The $\sigma^B$-dependent upregulation of *sar* expression observed in *rsbU*+ strains would be expected therefore to result in an increase in *agr* expression. However, the

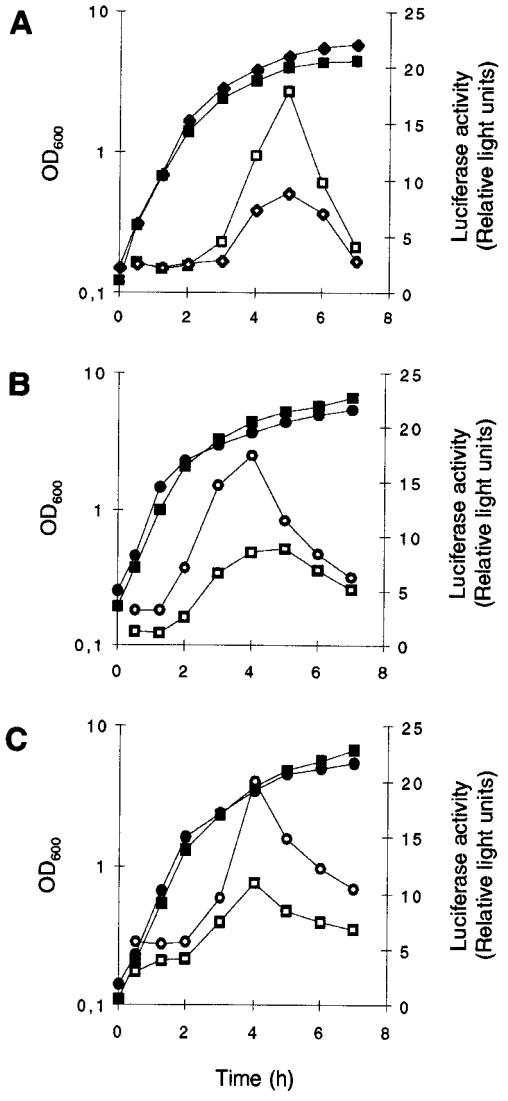


FIG. 7. Role of $\sigma^B$ in the regulation of *hldP*::*luc*+ expression during growth. *S. aureus* derivatives of strains BB255 (A), MSSA1112 (B), and Newman (C) were grown in LB medium at 37°C. Bacterial growth was measured by $OD_{600}$ (closed symbols). *hldp*::*luc*+ expression was determined by measuring the luciferase activity of the reporter gene *luc*+ (open symbols) as described in Materials and Methods. (A) Squares, *S. aureus* strain MB95 (BB255, *hldp*::*luc*+); diamonds, strain MB94 (BB255, *rsbU*$^+$ *hldp*::*luc*+). (B) Squares, *S. aureus* strain MB104 (MSSA1112, *hldp*::*luc*+); circles, strain MB112 (MSSA1112, *sigB hldp*::*luc*+). (C) Squares, *S. aureus* strain MB97 (Newman, *hldp*::*luc*+); circles, strain MB103 (Newman, *sigB hldp*::*luc*+).

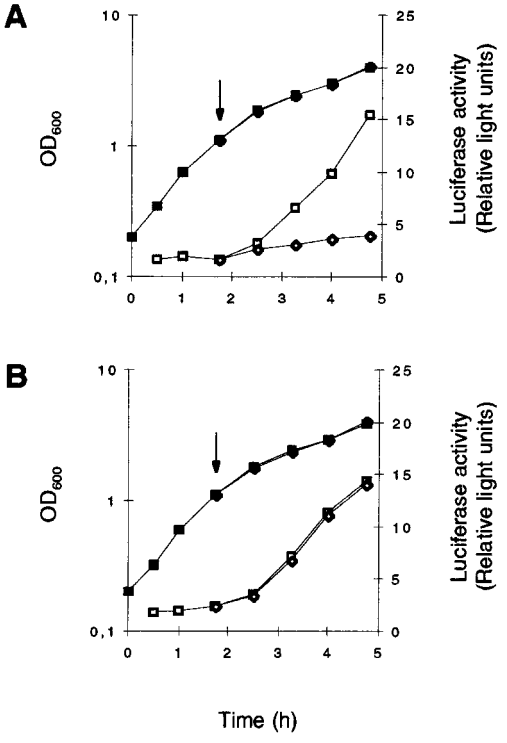


FIG. 8. Effect of overexpressed $\sigma^B$ on the expression of *hldP*::*luc*+. *S. aureus* derivatives of strain MB95 (BB255 *hldp*::*luc*+), harboring plasmid pIK64 ($P_{xyl}$::*sigB*) (A) or control plasmid pTX15 ($P_{xyl}$) (B), were grown in LB medium at 37°C. Growth was measured as the $OD_{600}$ (closed symbols). Growing cultures were split into equal parts at the $OD_{600}$ of 1, and overexpression of $\sigma^B$ was induced in one of the parts by supplementing 0.5% xylose (diamonds), while the control part was left without addition (squares). The arrow denotes the time point of supplementation. *hldp*::*luc*+ expression (open symbols) was determined by measuring the luciferase activity of the reporter gene *luc*+ as described in Materials and Methods.

data presented here revealed a weaker RNAIII transcription for the *rsbU*$^+$ strains MSSA1112 and Newman than for their Δ*rsbUVWsigB* mutants, suggesting that *agr* expression is negatively influenced by $\sigma^B$ activity, irrespective of the positive effect of $\sigma^B$ on *sar* expression. This finding is supported by a recent study of Chakrabarti and Misra (10), demonstrating an inhibitory influence of SarA on transcription from both the *agrP2* and *agrP3* promoters in vitro. The authors suggest that either SarA, together with an as-yet-uncharacterized cellular factor(s), activates transcription of the *agr* operon, or SarA regulates expression of one or more factors which then activate *agr* expression. In line with this hypothesis, this as-yet-uncharacterized cellular factor(s) involved in the activation of *agr* expression may be positively regulated by SarA but dominated negatively by $\sigma^B$ activity.

Many potential virulence factors have been shown to be regulated by SarA and RNAIII in a cooperative way (11, 13, 18, 27, 52), but the expression of several virulence factors was found to be upregulated by one regulator but repressed by the other (55, 60). Additionally, some virulence factors are influenced by one of the two loci but unaffected by the other one (7,

TABLE 2. Titration of fibronectin clumping of *S. aureus*

| Strain (genotype) | Fibronectin-clumping titer[a] | |
|---|---|---|
| | Exponential growth phase | Stationary growth phase |
| GP268 (*rsbU*$^+$*V*$^+$*W*$^+$*sigB*$^+$) | 1 | 1 |
| BB255 (*rsbU*) | 250 | 125 |
| BB255 (Δ*rsbUVWsigB*) | 125 | 125 |

[a] Clumping titer, lowest fibronectin concentration (in μg/ml) showing clumping. Results are expressed as the median of results from five to nine independent experiments.

26, 32). Thus, the $\sigma^B$-mediated increase in SarA, accompanied by the decrease in RNAIII, is very likely to enhance these phenomena, resulting in severe growth phase-dependent differences in the expression profiles of some virulence factors. One possible role of $\sigma^B$ in this scenario is to prolong the production of cell surface proteins, such as fibronectin binding proteins, that are positively influenced by SarA and negatively influenced by *agr* (55, 60). Simultaneously, $\sigma^B$ may down-regulate RNAIII-specific activities, i.e., the repression of protein A and upregulation of exoproteins (36, 49) or the production of capsular polysaccharides (22, 51). Consistent with this hypothesis is our finding that the *rsbU*$^+$ derivative GP268 possessed a significantly lower fibronectin-clumping titer than its *rsbU*-defective parent, BB255, signalling the presence of larger quantities of fibronectin binding proteins in GP268 than in BB255.

The impact of either SarA and/or RNAIII on the expression of virulence factors in *S. aureus* is well documented. On account of the studies performed with NCTC8325 derivatives, it is unquestionable that *sigB* mutants are still able to produce sufficient amounts of those two global regulators to be virulent, which led to the conclusion that $\sigma^B$ has no essential function in the virulence and pathogenicity of *S. aureus* (12). This conclusion is further strengthened by the findings of Nicholas et al. (47), who observed no differences between the clinical isolate WCUH29 and its isogenic Δ*sigB* mutant in their ability to cause infections in three distinct animal infection models. However, we agree with Gertz et al. (29), who question whether the infection models analyzed so far really reflect the natural situation in the host. The findings that both *agr* and *sar* expression are significantly influenced by $\sigma^B$ in an *rsbU*$^+$ genetic background should be reason to reevaluate if and how $\sigma^B$ is involved in the virulence and pathogenicity of *S. aureus*.

## ACKNOWLEDGMENTS

We are grateful to B. Berger-Bächi for critically reading and commenting on the manuscript. Preliminary sequence data was obtained from The Institute of Genomic Research (TIGR) through the website at http://www.tigr.org. Sequencing of *Staphylococcus aureus* COL was accomplished with support from National Institute of Allergy and Infectious Diseases (NIAID) and the Merck Genome Research Institute (MGRI).

This work was supported by the Swiss National Science Foundation grant NF 31-46762.96 to F. H. Kayser.

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