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σ^B Activity in a *Staphylococcus aureus hemB* Mutant

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Inactivation of *hemB* in *Staphylococcus aureus* strain Newman resulted in a small-colony phenotype and was accompanied by an altered expression pattern of global regulators and control of virulence factor production. Transcription profiles followed over 15 h by Northern blot analyses revealed that transcripts of the global regulators *arl*, *rot*, *sae*, *sarR*, *sarS*, *srr*, *svrA*, and *sigB* disappeared after the exponential phase and that both *agr* transcripts were completely absent in the *hemB* mutant. Apart from a general concentration of transcriptional activity to the exponential phase, premature gene expression was observed for *rot*, *hla*, and *spa*. Nevertheless, reported σ^B-dependent transcripts, such as *sarC* and *clfA*, were produced throughout the 15-h growth period monitored. The absence of these transcripts in a *hemB sigB* double mutant demonstrated their dependence on σ^B and indicated an unexpected, permanent σ^B activity in the *hemB* mutant. Variations in the extents of the directly σ^B-controlled *asp23*, *rsbVW-sigB*, and *sarC* transcripts argue for additional factors modulating σ^B activity. This study provides the first extended synopsis of the transcriptional patterns of different regulators over the entire growth cycle in the widely used Newman strain.

Staphylococcus aureus is an opportunistic pathogen and one of the major causes of nosocomial and community-acquired infections. Adaptation to changing conditions and a wide spectrum of diseases ranging from superficial to serious life-threatening infections and toxicoses is conferred by a formidable arsenal of pathogenicity and virulence factors. Besides their remarkable ability to acquire multiple resistance determinants, staphylococci adopt additional strategies to evade antibiotic challenge and host defenses. The formation of small-colony variants (SCVs) and the capacity of these SCVs to persist intracellularly are regarded as survival mechanisms employed by staphylococci (55). SCVs are recovered from clinical specimens, particularly from patients with chronic, persisting, and/or relapsing infections (54, 61). Characteristically, SCVs grow slowly and form tiny, unpigmented, nonhemolytic colonies on solid medium. Clinical SCVs are often auxotrophic for hemin or menadione (1, 61), compounds involved in the synthesis of the electron carriers cytochrome and menaquinone, respectively, and therefore have an impaired electron transport. Disruption of the *hemB* gene, encoding the essential aminolevulinic acid dehydratase for hemin biosynthesis, leads to a stable *S. aureus* model of SCV, avoiding undefined genetic backgrounds and reversible phenotypes, which occur in clinical SCVs (3, 10, 31, 58, 66).

An interrupted electron transport chain causes accumulation of the reducing equivalents NAD(P)H and $FADH_2$, a low membrane potential, and low ATP concentration (34, 53). Metabolic pathways dependent on the availability of the oxidizing equivalents $NAD(P)^+$ and FAD are inhibited, which reduces the range of carbohydrate utilization. Several genes involved in glycolysis and fermentation are up-regulated in a COL *hemB* mutant, whereas tricarbonic acid cycle enzymes are down-regulated, manifesting divergent metabolic activity from that of the wild type and leading to a lower energy production (34). Consequently, the ATP-requiring biosynthesis of proteins, cell wall, or nucleotides is limited and finally cell growth stalls. However, the reduced transmembrane potential protects *hemB* mutants and clinical SCVs from cationic antibiotics (3), slow growth lowers the efficacy of substances targeting metabolically active cells, and the low toxin production allows them to persist in the host cell, which is a relatively protected environment (2).

Controlled and coordinate expression of virulence determinants during *S. aureus* infection is regulated by a multitude of global regulators (12, 48). The major accessory gene regulator locus *agr* represses via two divergently transcribed mRNAs (RNAII and RNAIII) in post-exponential phase cell-surface associated proteins, such as protein A (*spa*) or fibronectin-binding protein A (*fnbA*), and activates exotoxin production, e.g., α-toxin (*hla*). RNAII encodes a quorum-sensing two-component system, which upon increasing cell density induces the expression of the *agr* effector molecule RNAIII (29, 45, 50, 60). Staphylococcal accessory regulator A (*sar*) encodes three overlapping and differentially regulated transcripts (*sarB*, *sarC*, and *sarA*). While *sarB* and *sarA* are σ^A dependent and transcribed mainly during exponential growth, the σ^B-dependent *sarC* transcript appears later and increases towards stationary phase. However, all three transcripts encode the global regulator SarA (4, 15, 39), positively influencing *fnbA* and *hla* (13, 68) but inhibiting *spa* expression (20). σ^B, an alternative sigma factor mediating stress response, controls target genes via σ^B-dependent promoters, as in the case of *sarC*. Indirectly, σ^B inhibits *agr* and *hla* (7, 8, 13, 27); on the other hand, σ^B positively influences *fnbA* expression (7, 46). Additional two-component systems and transcription factors take part in a complex regulatory network, further modulating the above-described regulators and virulence determinants. As a result, in

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TABLE 1. Primers used for amplification of specific digoxigenin-labeled DNA probes[a]

Probe	Primer	Sequence (5′–3′) and reference	Probe	Primer	Sequence (5′−3′) and reference
arlS	MS43	GATAACACAGTGAGAGTTGAACC	*saeR*	MS45	GACCCACTTACTGATCGTG
	MS61	GAGTCCATTACCGCCTTGAC		MS46	CCTAATCCCCATACAGTTGTG
asp23	SA*asp*23f	ATGACTGTAGATAACAATAAAGC (23)	*sar*	S*asarA*f	AGGGAGGTTTTAAACATGGC (11)
	SA*asp*23r	TTGTAAACCTTGTCTTTCTTGG (23)		S*asarA*r	CTCGACTCAATAATGATTCG (11)
clfA	MS33	CGTGGCTTCAGTGCTTGTAG	*sarR*	*sarR*$^+$f	CTTCTAATTCTGAAATCAG (57)
	MS34	GAGTTGTTGCCGGTGTATTAGC		*sarR*r	GACATTAATGATTTAGTCAAC (57)
fnbA	MS16	GGGATGGGACAAGATAAAGAAGC	*sarS*	MS42	CAAGCCTGAAGTCGATATGAC
	MS17	ACGACACGTTGACCAGCATG		MS41	CAGCATGGTCTTGCTGC
hla	MS20	AGAAAATGGCATGCACAAAAA	*sigB*	IK14	ACGCGAAGGTGGCCTAG (35)
	MS21	TGTAGCGAAGTCTGGTGAAAA		IK15	ATGGTCATCTTGTTGCCCC (35)
isaA	MS75	GGCATCATCATTAGCAGTGG	*spa*	*spa*f	TGAATTCGTAAACTAGGTGTAGG (57)
	MS74	GAATCCCCAAGCACCTAAAC		*spa*r	CGGTACCAGGCTTGTTATTGTCTTCC (57)
RNAII	MS14	CGAAGACGATCCAAAAC	*srrA*	MS59	CGAAATACTTATCGTAGATGATGAGGATAG
	MS15	TTATCTAAATGGGCAATGAGT		MS60	CAGCAAGTACGCGATGTGC
RNAIII	*RNAIII*f	GTGATGGAAAATAGTTGATGAG (11)	*svrA*	MS31	CATTGCCAATGATGATAGGGAC
	*RNAIII*r	GTGAATTTGTTCACTGTGTCG (11)		MS64	CATTGCTGCTAAAGCACAAAG
rot	MS39	CAAGTTTTGGGATTGTTGGGATG (adapted from reference 59)	*yycFG*	MS67	GAAGGATACGATGTGTACTGTGC
	MS40	GCTCCATTCATTTGTGCCATAG		MS68	CGTTTCGACCTCTACTCATGTTG

[a] f, forward, r, reverse.

vitro, wild-type *S. aureus* first expresses surface-associated proteins, such as FnbA, followed by secreted protective proteins, like protein A, as well as exoenzymes, exemplified by α-toxin. Thereby, the transition from a colonizing to an invasive behavior is represented in a growth-phase-dependent manner (56). *hemB* mutants were found to express protein A and α-toxin only weakly (31, 66), whereas the adhesion factor FnbA and its homologue FnbB, as well as the fibrinogen-binding clumping factor A (ClfA), were up-regulated (65). The surface-associated virulence determinants FnbA and ClfA are important for colonization, escape from host defense, and persistence (44, 52, 62). Also of interest, *clfA* expression requires the alternative sigma factor σ^B (18, 46, 47), for which reason it seemed likely that this stress response regulator may be active in *hemB* mutants. However, the overall *sar* mRNA levels, including that of the σ^B-dependent *sarC* transcript, were reported to be reduced in the 8325-4 *hemB* mutant (65). Since no distinction was made between the three overlapping, SarA encoding, but differentially regulated *sarB*, *sarC*, and *sarA* transcripts originating from the *sar* locus, σ^B activity in *hemB* mutants remained open.

In addition to σ^B, none of the staphylococcal regulators have been analyzed in *hemB* mutants in detail. We therefore investigated in this study the expression not only of σ^B but also of most known global regulators and selected virulence determinants in strain Newman and its *hemB* mutant over an extended time period. As several of the analyzed loci have multiple, differently controlled promoters, Northern blot analyses were preferred to reverse transcription-PCR techniques to conveniently identify individual transcripts. For monitoring σ^B activity in the *hemB* mutant, a luciferase reporter construct under the control of a σ^B-dependent promoter was used. To determine the impact of σ^B absence, a *hemB sigB* double mutant was constructed and the expression profiles of σ^B-dependent transcripts were compared to those in the *hemB* background.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains were constructed in a Newman background (17). Strain MS17 was obtained by transducing the *hemB*::*ermB* mutation with phage 85 (5) from the strain 8325-4 derivative I10 (66) into the parent strain MB79, which is a Newman strain with an integrated reporter construct carrying the firefly luciferase gene (*luc*$^+$) fused to the three σ^B-dependent alkaline shock protein 23 promoters (*asp23P*::*luc*$^+$) (23). Strain MS62 was constructed by transducing the *tetL*-linked *sigB1*(Am) mutation from the 8325-4 derivative GP266 (6) into strain III33, a *hemB*::*ermB* mutant of strain Newman (31). The constructs were confirmed by PCR, Southern blot analysis, and SmaI–pulsed-field gel electrophoresis chromosomal pattern.

Bacteria were grown aerobically at 37°C. Agar plates containing Luria-Bertani broth (LB), Columbia blood agar base with sheep blood, or Mueller-Hinton (MH) broth were used for cultivation on solid media. Liquid cultures were grown in LB, and good aeration was assured by vigorously shaking flasks with an air-to-liquid ratio of at least 4. Transductants were selected on 10 μg/ml tetracycline and 2.5 μg/ml erythromycin. Hemin (1 μg/ml) was used to supplement *hemB* mutants where needed (66).

Antibiotic susceptibilities were determined with Etest strips (AB-Biodisk, Solna, Sweden), covering an exponential gradient ranging from 0.016 to 256 μg/ml, on MH agar plates with an inoculum of a 0.5 McFarland standard, corresponding to 10^8 cells/ml. MICs were read after 24 h of incubation and, in the case of the mutants, after 24, 48, and 72 h, during which time MICs did not change.

To sample RNA, protein, and luciferase probes, cells from overnight cultures were washed in LB at 37°C and used to inoculate prewarmed LB to an optical density at 600 nm (OD_{600}) of 0.1, corresponding to a concentration of 10^7 cells/ml. After 15 h, CFU as well as phenotype were determined on blood agar and selective plates to assure that no contamination or reversion had occurred during the experiment.

Northern blot analyses. Total RNA was isolated as described previously (14) by using a FastRNA kit and a Fastprep reciprocating shaker (Bio 101, Vista, Calif.). For Northern blots, 5 to 10 μg of total RNA per lane was separated on a 1.5% agarose-20 mM guanidine thiocyanate gel and transferred overnight onto a positively charged nylon membrane (Roche, Rotkreuz, Switzerland). The blots were hybridized with specific digoxigenin-labeled DNA probes which were produced using a PCR DIG probe synthesis kit (Roche, Rotkreuz, Switzerland). Primers used are listed in Table 1. Membranes were stripped by being boiled twice in 0.1 SSC-0.5% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min and were reprobed up to four times. Data shown were confirmed in at least two independent experiments.

Western blot analyses. Pelleted bacteria were resuspended in 0.07 M phosphate buffer, pH 6.8, containing lysozyme, lysostaphin, and DNase (each at 0.018 mg/ml) as well as 2 mM phenylmethylsulfonyl fluoride. Protein (10 μg) from cytoplasmic fractions was loaded for each lane and separated by SDS-10% polyacrylamide gel electrophoresis. Precision Plus Protein all blue standards (Bio-Rad) were used as molecular size markers. Gels were either stained with Coomassie blue dye (R25; Réactifs IBF, Villeneuve-la-Garenne, France) or transferred onto nitrocellulose membranes (Hybond; Amersham Biosciences). Rabbit anti-Asp23 antibodies were used to detect Asp23. For σ^B detection, blocked membranes were preincubated with 40 μg/ml human immunoglobulin G (Calbiochem) to saturate protein A and thereby prevent cross-reactivity of an-

tigen-purified rabbit antibodies against σ^B. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:10,000 and detected with SuperSignal West Pico solutions (Pierce). Representative blots from three independent experiments are shown.

Luciferase assay. Bacteria were harvested by centrifugation, and the pellet was snap-frozen in liquid nitrogen. Cells were resuspended to an OD_{600} of 10, corresponding to 10^9 cells/ml, in 0.7 M phosphate buffer, pH 6.8, containing lysozyme and lysostaphin (each at 0.036 mg/ml). After shaking for 10 min at 37°C, 10 μl supernatant of lysed cells was mixed with an equal volume of luciferase assay substrate (Promega) and luminescence was measured for 15 s after a delay of 3 s on a Turner Designs TD-20/20 luminometer (Promega). According to the manufacturer's information, the luciferase assay substrate contains excess ATP; addition of 2 mM ATP did not increase signals. Protein concentration of the supernatant was determined by the Bradford method (9), with bovine serum albumin as a standard. Representative data from three independent experiments are shown.

Determination of mRNA stability. Bacteria grown for 5 h in LB were supplemented with 300 μg/ml rifampin, and samples were taken at 3-min intervals. Total mRNA was isolated and analyzed by Northern blotting, as described above. Band intensities were quantified densitometrically with an ImageMaster VDS-CL (Amersham Pharmacia Biotech) using ImageQuant version 5.2. Values were corrected against the background and normalized. Half-life of mRNA was determined from regression lines obtained by plotting mean values of at least two independent experiments against time on a semilogarithmic graph. The regression line was calibrated to intercept with the initial amount of transcripts, which was set to 100.

RESULTS

Phenotypes and growth characteristics of the *hemB* mutants. To evaluate the importance of σ^B in *hemB* mutants, strain MS17, carrying a σ^B-dependent luciferase reporter fusion (23), and the reporterless *hemB sigB* double mutant MS62 were constructed. Both mutants formed tiny colonies on MH plates, whereas on sheep blood agar, cells could scavenge enough hemin to form wild-type-sized, hemolytic colonies. In LB, they reached a maximal OD_{600} of 0.8, while their parent, MB79, grew up to an OD_{600} of 10 (Fig. 1A, data not shown for MS62). Viability was not reduced in the *hemB* mutants, as judged from CFU determined over several days, which corresponded to the respective OD_{600} values (Fig. 1B and C). Both mutants were four and eight times more resistant to the aminoglycosides gentamicin and amikacin, respectively. However, they were slightly more susceptible to oxacillin and teicoplanin by factors of 2 and 4, respectively, but not to vancomycin (Table 2). By supplementing the growth medium with hemin, all phenotypes reported here and further could be restored to a wild-type pattern in the mutants, indicating that inactivation of *hemB* was solely responsible for the characteristics of the mutants and that no unexpected mutations or rearrangements had occurred during the construction of the strains.

In cultures with an initial OD_{600} of 0.01, the lag phase of *hemB* mutants was more than 4 h longer than that of the parent strain (Fig. 1A). Cultures were therefore generally started with an inoculum of OD_{600} of 0.1, which synchronized the beginning of exponential growth of wild-type bacteria and *hemB* mutants (Fig. 1C). Under these conditions, the mutants entered the stationary growth phase 2 h later than the parent strain. Carryover of extracellular signal molecules, which might mask growth-dependent processes, was avoided by washing cells before inoculation. Growth was followed over 15 h to include late-stationary-phase data.

Transcription of two-component systems is restricted to the exponential phase in the *hemB* mutant. Expression patterns

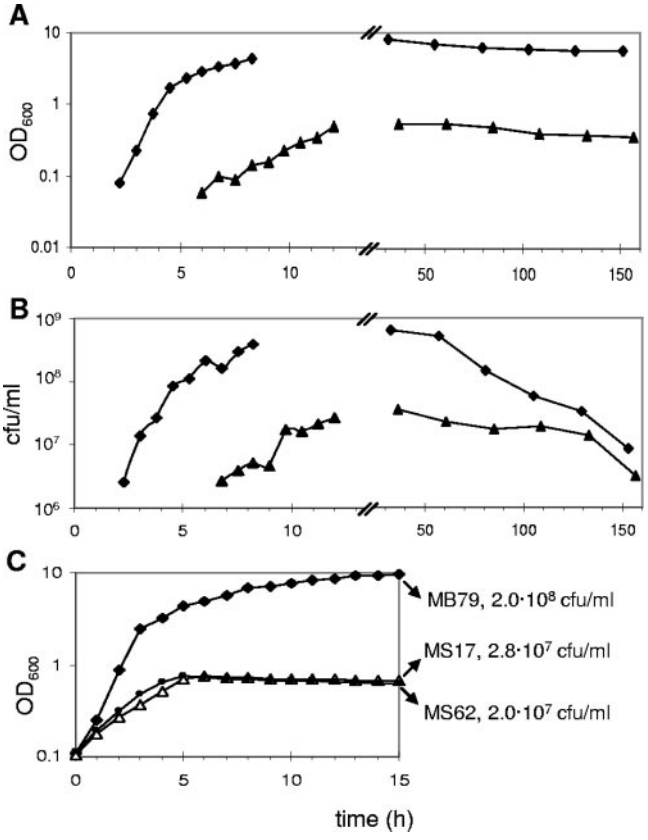

FIG. 1. Growth and viability of MB79 (wild type), MS17 (*hemB*), and MS62 (*hemB sigB*). (A) Growth curves and (B) CFU of cultures, inoculated with overnight cells and an initial OD_{600} of 0.01, were monitored over 6 days. (C) Growth curves from cultures with an inoculum 10 times higher (OD_{600} of 0.1) than that for panels A and B, obtained by using washed overnight cells. CFU/ml were determined after 15 h. MB79, diamonds; MS17, triangles; MS62, circles.

regarding *agr*, *arl*, *sae*, and *srr* were in agreement with previous studies of the wild type, MB79 (Fig. 2) (19, 24, 49, 63, 70). The 2.7-kb *arlRS* transcript, coding for an autolysis-related sensor transducer involved in biofilm formation, was seen throughout growth and increased slightly in the late stationary phase. Of the three *sae* transcripts, *saePQRS* (3.1 kb), *saeQRS* (2.4 kb), and *saeRS* (2 kb), whose products influence several virulence factors presumably in response to environmental stimuli, *saeQRS* was the most prominent and peaked in the late post-exponential phase. The weaker 2.5-kb *srrAB* (synonym, *srhSR*) and the generally stronger 0.7-kb *srrA* mRNAs, reported to be produced until post-exponential phase and encoding a system

TABLE 2. Antibiotic susceptibilities of strains MB79 (wild type), MS17 (*hemB*), and MS62 (*hemB sigB*)

Antibiotic	MIC (μg/ml) of:		
	MB79	MS17	MS62
Amikacin	4	32	32
Gentamicin	1	4	4
Oxacillin	0.38	0.19	0.19
Teicoplanin	3	0.75	0.75
Vancomycin	2	2	2

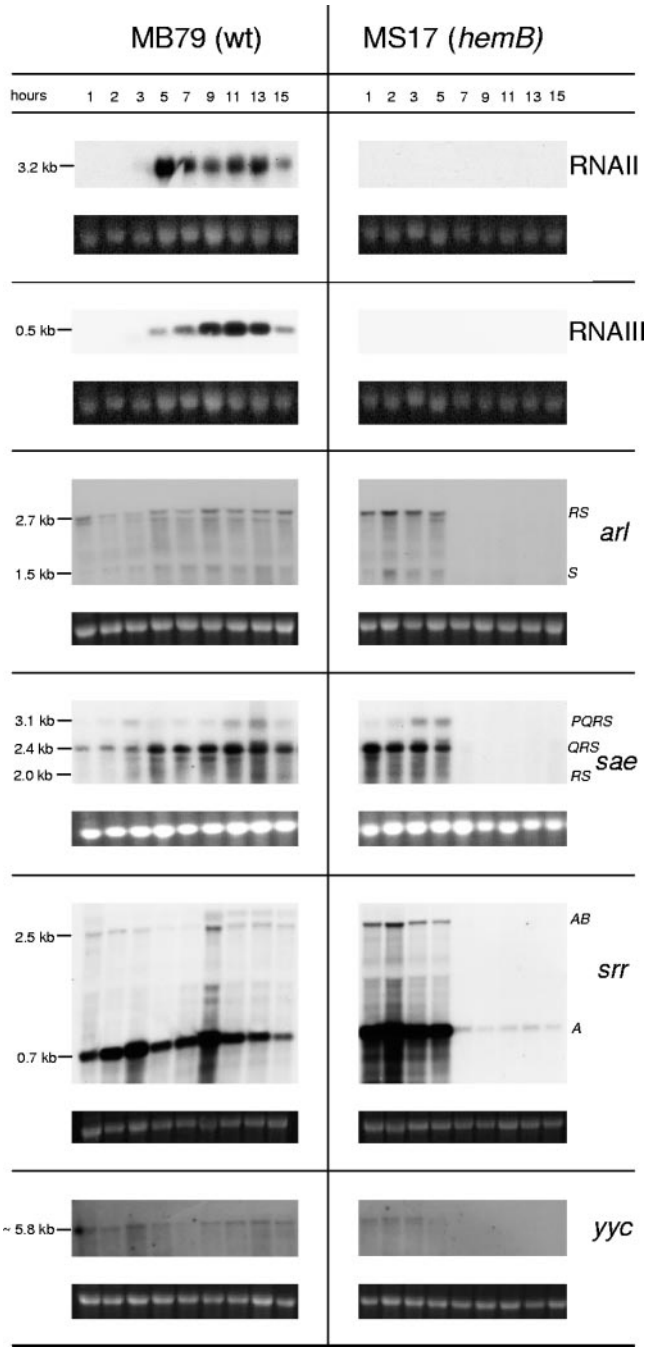

FIG. 2. Northern blot analyses of the two-component systems *agr* (RNAII and RNAIII), *arl*, *sae*, *srr*, and *yyc* in MB79 (wild type [wt]) and MS17 (*hemB*). Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading.

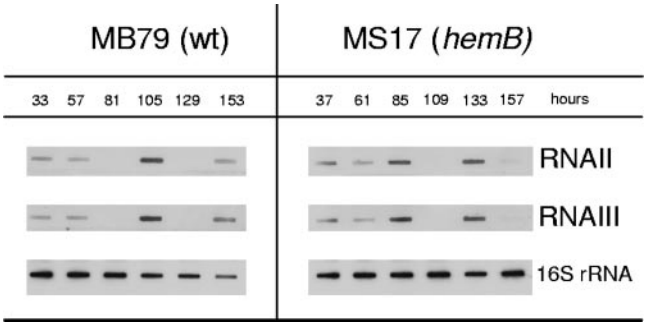

FIG. 3. *agr* expression during prolonged cultivation. MB79 (wild type [wt]) and MS17 (*hemB*) were monitored over 7 days. RNA samples were taken every 24 h, prepared as described in Materials and Methods, and transferred onto a nylon membrane by slot blotting. Bacteria were grown as described in the legend for Fig. 1A and B.

reacting to environmental oxygen changes, showed here peaks at 3 and 9 h and were expressed throughout the 15 h monitored. The expression profile of *yycFG* (synonym, *vicRK*), coding for an essential two-component system affecting cell permeability as well as resistance against macrolide-lincosamide-streptogramin B antibiotics (16, 41), was similar to that of *arl*. A transcript of approximately 5.8 kb was observed, suggesting that *yycFG* was cotranscribed with at least two of the yet uncharacterized downstream genes.

Transcripts of *arl*, *sae*, *srr*, and *yyc* disappeared in the *hemB* mutant after 5 h, except for a very faint *srrA* band detected throughout growth. In this short period of expression, *arl*, *sae*, and *srr* transcription levels were up-regulated in the *hemB* mutant compared to levels in MB79, while *yyc* was expressed at similar levels (Fig. 2). In the Newman *hemB* mutant MS17, unlike as reported for a *hemB* mutant in a strain 8325-4 background (65), not only was the *agr* transcript RNAIII (0.5 kb) absent, but RNAII (3.2 kb) was missing as well (Fig. 2). Supplementation of the growth medium with hemin restored the *agr* profile in MS17 to the wild-type pattern, ruling out an unrelated deletion or mutation affecting *agr* (data not shown). Interestingly, preliminary long-term monitoring revealed both *agr* transcripts to be expressed after 30 h in the *hemB* mutant MS17 (Fig. 3). As in the parent MB79, *agr* transcription fluctuated in MS17, stopping after 3 or 4 days, respectively, to resume at day 5 or 6, respectively. A second transcriptional gap was observed for both strains after an additional day, reminiscent of the oscillating *agr* expression levels observed with *S. aureus* biofilms (69), and here might be caused by growth of a new subpopulation living on dead cells and debris once nutrients had been depleted from the medium.

Expression of *agr* requires *svrA*, encoding a putative membrane-associated protein affecting virulence (21), which recently has been identified to be a multidrug export protein (therefore renamed *mepA*) belonging to the MATE family of efflux pumps (42). The transcript(s) of yet unknown size, reported to be expressed in post-exponential phase (21), were here however detected only during early growth in the MB79 wild type. A faint band of approximately 2.5 kb suggested that *svrA* (1.35 kb) is cotranscribed with two flanking genes, encoding a MarR-like transcriptional regulator (*mepR*) and a hypothetical protein of yet-unknown function (*mepB*) (Fig. 4). A shorter, approximately 1.5-kb transcript might be present, paralleling the larger transcript. In MS17, the transcripts were almost undetectable.

Reduced transcription of SarA homologues in the *hemB* mutant. The SarA family of transcriptional regulators constitutes a further set of major regulatory elements. They include Rot, SarA, SarR, SarS, SarT, and SarU (12). *sarT* and *sarU*, not

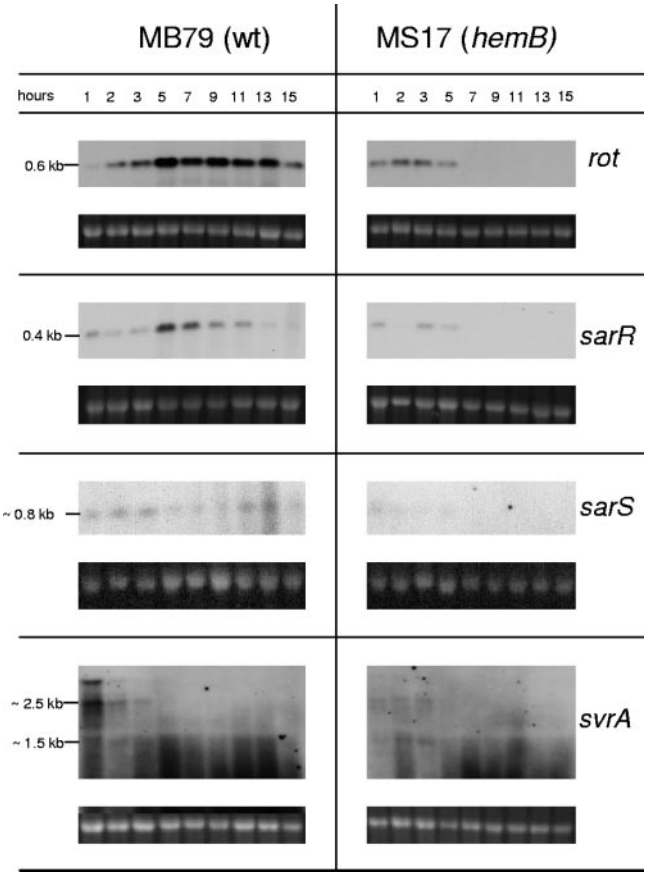

FIG. 4. Northern blot analyses of the SarA homologues *rot*, *sarR*, and *sarS* as well as the virulence regulator *svrA* transcripts in MB79 (wild type [wt]) and MS17 (*hemB*). Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading. The observed band at the height of approximately 2.8 kb in *svrA* blots might be caused by interference of bulk 23S rRNA.

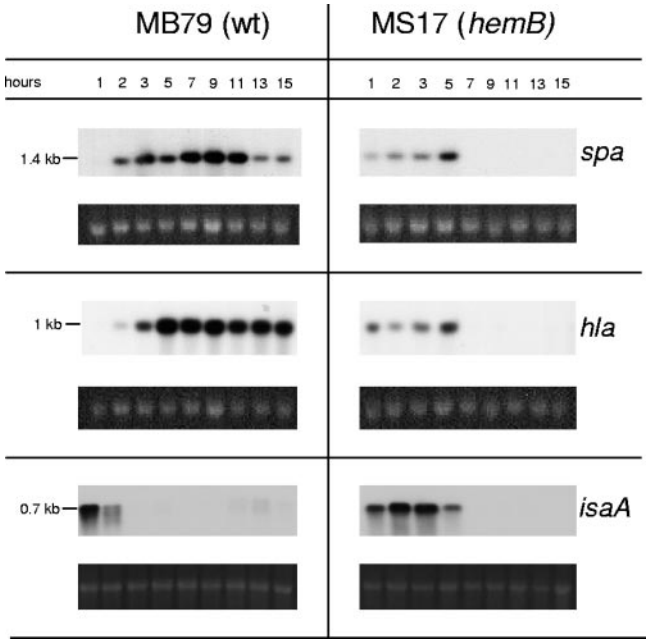

FIG. 5. Northern blot analyses of the virulence determinants *spa*, *hla*, and *isaA* in MB79 (wild type [wt]) and MS17 (*hemB*). Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading.

being expressed in the Newman background according to microarray data (M. Bischoff, unpublished data), were not analyzed here.

In MB79, both *rot* (0.6 kb) and *sarR* (0.4 kb) were transcribed maximally in the post-exponential phase, only to decline towards the stationary phase (Fig. 4), as reported previously (38, 67). The *sarS* transcript (0.8 kb) showed an antiparallel behavior, being present in low amounts in early exponential phase (64) and then again towards stationary phase. In contrast to these wild-type patterns, the *hemB* mutant MS17 displayed reduced transcription levels of *rot*, *sarR*, and *sarS* and only during exponential phase (Fig. 4).

Virulence determinants *spa*, *hla*, and *isaA* are transcribed only in exponential phase in the *hemB* mutant. Besides complex mutual interactions, the above-presented regulators, together with the below-discussed SarA and σ^B, affect virulence gene expression directly or indirectly. From the transcriptional data presented here, it was possible neither to predict the amount of active regulator nor to attribute net effects to a single regulator. The different resulting transcription patterns are exemplified for the virulence factors *spa*, *hla*, and *isaA*.

In MB79, *spa* was maximally transcribed in post-exponential phase; *hla* transcription started shortly after that of *spa* but reached its maximum level earlier and was thereafter rather constant (Fig. 5). Overall transcription levels of both *spa* and *hla* were reduced in the *hemB* mutant MS17, in agreement with previous findings (66); yet, surprisingly we observed that transcription started earlier than in the parent (Fig. 5), a phenomenon also seen for *rot* (Fig. 4).

Transcription of the immunodominant antigen A (*isaA*), whose product had previously been reported to be up-regulated and present in stationary growth phase in a COL *hemB* mutant (34), was prolonged and increased in MS17 compared to transcription in MB79 (Fig. 5). However, transcription stopped upon entry into stationary phase, as in the case of *spa* and *hla*.

σ^B expression and activity in the *hemB* mutant. σ^B mediates various stress responses and also plays a role in antibiotic resistance as well as biofilm formation (summarized in reference 7). Transcription of the *sigB* operon is presumably initiated by three promoters, producing a 3.6-kb transcript containing *sas067-sa1873-rsbUVW-sigB* (P1-*sigB*), an *rsbUV-sigB*-covering 2.5-kb transcript (P2-*sigB*), and a third, 1.6-kb, stress-inducible, autoregulated transcript covering *rsbVW-sigB* (P3-*sigB*) that is σ^B dependent (22, 23). In strain MB79, the P1- and P2-driven mRNAs were expressed weakly and only during early growth, while the 1.6-kb P3-*sigB* transcript displayed a more complex pattern: it peaked in late exponential phase and in early post-exponential phase and increased again towards stationary phase (Fig. 6B). In contrast, in the *hemB* mutant MS17, the P1- and P2-driven transcripts were almost undetectable and the 1.6-kb P3-*sigB* transcript, initially stronger than in the parent, disappeared after the exponential phase.

Despite the difference in transcriptional activities, Western blot analyses of cytoplasmic protein fractions of the MB79 wild

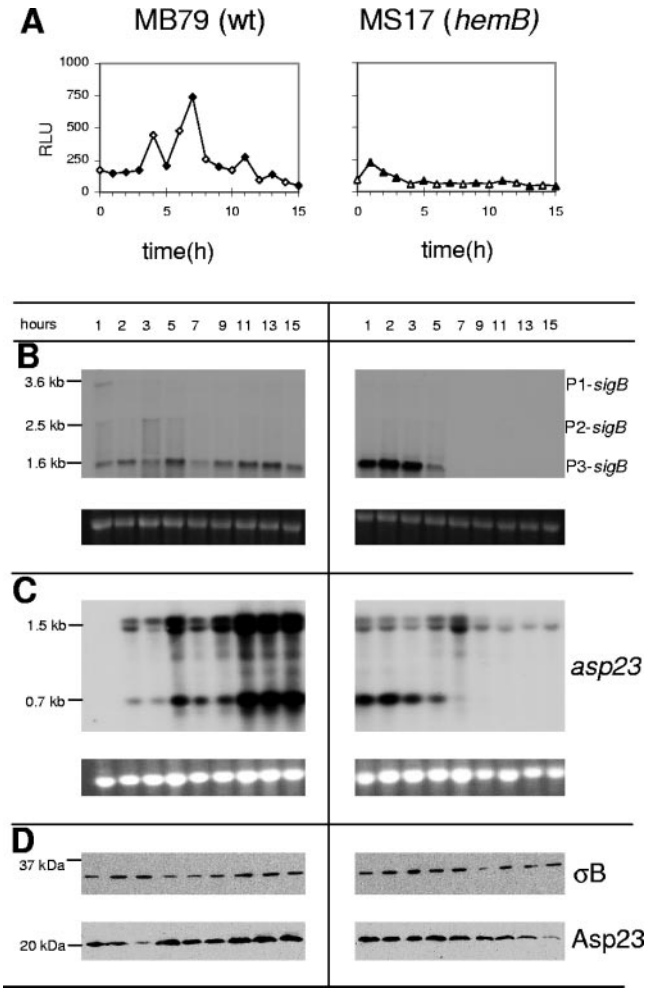

FIG. 6. σ^B expression in the Newman background. (A) σ^B activities in MB79 (wild type [wt]) and MS17 (*hemB*). σ^B activities were measured by the σ^B-dependent *asp23P*::*luc*$^+$ reporter gene fusion. Luciferase activity is given as RLU per μg protein of cleared lysate. Filled symbols indicate time points of sampling for Northern and Western blot analyses. (B and C) Northern blot analyses of (B) *sigB* and of (C) *asp23*. The sizes of relevant bands are given on the left. Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading. The observed double band at the site of the 1.5-kb *asp23* mRNA might be caused by interference of bulk 16S rRNA. (D) Western blot analyses of σ^B (~35 kDa) and Asp23 (~23 kDa). Cytoplasmic protein fractions were separated by SDS-10% polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and analyzed with antigen-purified anti-σ^B antibodies (upper panels) or anti-Asp23 antibodies (lower panels). The molecular sizes of the Precision Plus Protein all blue standard (Bio-Rad) markers are indicated on the left.

type and the *hemB* mutant MS17 revealed that the σ^B protein was present at similar, constant levels in both strains throughout growth (Fig. 6D). Presence of the σ^B protein, however, is not necessarily indicative of its activity, due to posttranslational control by RsbU, RsbV, and RsbW (23, 43, 51). A reporter construct consisting of the σ^B-dependent promoters of the alkaline shock protein 23 (*asp23*) fused to the firefly luciferase gene (*luc*$^+$) was therefore used to analyze the actual σ^B activity. The luciferase activity profile followed the peaks of the 1.6-kb P3-*sigB* transcript in MB79 with an approximately-1-h delay (Fig. 6A). In the *hemB* mutant MS17, σ^B activity could be detected only during the first 2 h of growth, where relative light unit (RLU) values were higher in the *hemB* mutant than in MB79. Thereafter, RLUs dropped quickly below the values measured in the parent (Fig. 6A). Western blot analysis showed that Asp23, which was here under the same control as luciferase, decreased in the mutant over the time course as well, while in MB79, Asp23 seemed to increase from the end of the exponential phase towards stationary phase (Fig. 6D).

The resident *asp23* gene is preceded by two σ^B consensus sequence promoters producing 0.7- and 1.5-kb transcripts with identical 3′ ends (23). In the parent strain MB79, both *asp23* transcripts paralleled the expression profile of the 1.6-kb P3-*sigB* mRNA, peaking at 5 h and reaching maximal levels during the last 3 h (Fig. 6B and C). In the *hemB* mutant, however, only the 0.7-kb *asp23* mRNA paralleled the *hemB* mutant-specific 1.6-kb P3-*sigB* transcription profile, with transcription stopping after 5 h of growth. Surprisingly, the upper *asp23* transcript was present at low levels throughout the 15 h. Contrary to what the 1.6-kb P3-*sigB* mRNA and luciferase measurements had suggested, these data indicated a possible σ^B activity in the mutant. We therefore compared transcription levels of other known σ^B-controlled genes with the expression levels in the *hemB* mutant MS17 and the *hemB sigB* mutant MS62.

Transcription of the σ^B-influenced genes *sarC*, *clfA*, and *fnbA* requires σ^B in the *hemB* background. For MB79, we observed characteristic *sar* profiles (4). *sarA* (0.5 kb) and a weak *sarB* (1.2-kb) transcript were mainly present in exponential phase, while the σ^B-dependent *sarC* (0.8-kb) transcript appeared in mid-exponential phase, reaching a maximum intensity in post-exponential phase (Fig. 7). Interestingly, in the *hemB* mutant MS17, all three *sar* transcripts were maximally expressed in the beginning and were strongly increased compared to in MB79. While *sarA* and *sarB* were restricted to the exponential phase, *sarC* was transcribed throughout growth, although slightly decreasing towards stationary phase. We confirmed the *sarC* transcript to be σ^B dependent in the *hemB* background as well, since it was abolished in the *hemB sigB* double mutant MS62 (Fig. 7).

The *clfA* gene is preceded by σ^A- and σ^B-dependent promoters, which initiate transcripts of 3.7 kb and 2.9 kb, respectively (18, 46). The 3.7-kb σ^A-dependent transcript was seen in all three strains during exponential growth phase, irrespective of the changes triggered by *hemB* or *sigB* inactivation (Fig. 7). The σ^B-dependent 2.9-kb *clfA* mRNA roughly followed the 1.6-kb P3-*sigB* transcription profile in MB79, with peaks at 3 h and 13 to 15 h. In the *hemB* mutant MS17, however, the 2.9-kb *clfA* transcript displayed a transcriptional pattern quite different than that of the 1.6-kb P3-*sigB* mRNA. After a strong initial signal, transcription diminished slightly and then increased again towards stationary phase, comparable to what was observed for the parent strain. The σ^B dependence of the 2.9-kb *clfA* mRNA was confirmed by its absence in the *hemB sigB* double mutant MS62.

Although not preceded by an apparent σ^B consensus promoter sequence, the *fnbA* gene is positively influenced by σ^B (7, 18, 46). While the recently reported σ^A-dependent 4.5-kb *fnbA* transcript (18) was not observed in the Newman background, a 3.1-kb *fnbA* transcript was detected during the first hours after inoculation in MB79 and MS17 but was completely

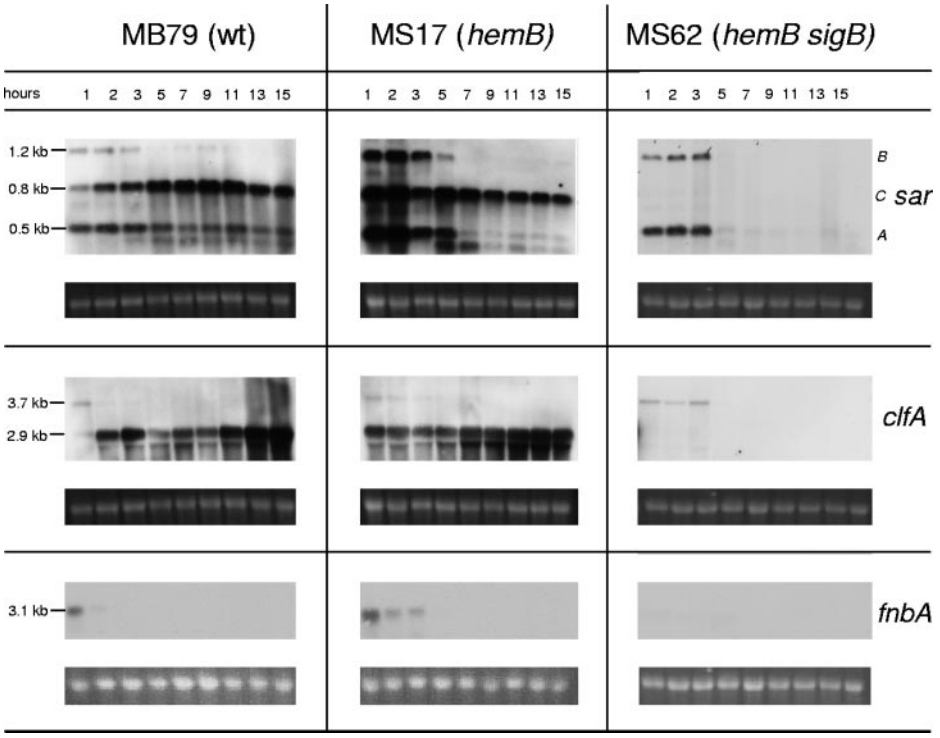

FIG. 7. Northern blot analyses of the σ^B-influenced genes *sar*, *clfA*, and *fnbA* in MB79 (wild type [wt]), MS17 (*hemB*), and MS62 (*hemB sigB*). Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading.

missing in the *hemB sigB* mutant MS62 (Fig. 7). In the *hemB* mutant MS17, *fnbA* transcription was slightly prolonged compared to in the parent strain MB79.

The presence of σ^B-dependent *asp23*, 2.9-kb *clfA*, *fnbA*, and *sarC* transcripts in the *hemB* mutant MS17 and their respective absences in the *hemB sigB* mutant MS62 (data not shown for *asp23*) proved that σ^B was required and active at all time points in MS17. Nevertheless, in this strain, striking differences in the extents of stimulation of the directly σ^B-regulated genes were observed. Variations in mRNA half-lives of these σ^B-dependent transcripts were ruled out. There was no significant difference in mRNA stability between parent and mutant or between *sarC* and 2.9-kb *clfA* (Fig. 8). The approximate half-life of transcripts was 3 min, similar to values determined for the *asp23* transcripts (Bischoff, unpublished). Therefore, variations in regulation of directly σ^B-controlled promoters might be attributed to the involvement of additional factors modulating σ^B activity.

DISCUSSION

Differences in genetic backgrounds as well as minor changes in growth conditions can have major impacts on the profiles of regulators. Comparison of wild-type and growth-deficient strains, like *hemB* mutants, is in addition complicated by a growth phase disparity. By starting cultures with a relatively high inoculum of washed cells from overnight cultures, lag phases were synchronized (Fig. 1C), having no influence on the growth phase-dependent patterns of gene expression, as judged from *agr* and *sar* profiles (Fig. 2 and 7). Cells were sampled frequently enough to obtain representative profiles for both strains and to allow comparison of the temporal gene expression profiles from the respective growth phases. Hence, this study provides for the first time a useful overview of wild-type and *hemB* mutant transcription profiles of the most important regulators.

We analyzed the temporal patterns of several global regulators for 15 h and found that their transcriptional profiles did

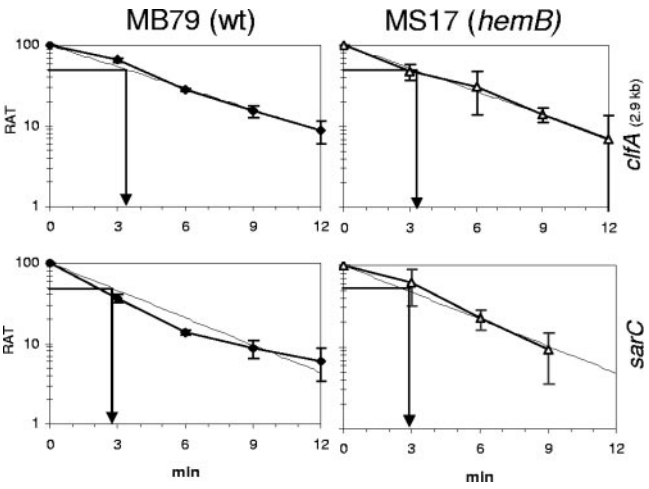

FIG. 8. Stability of 2.9-kb *clfA* and *sarC* mRNAs in MB79 (wild type [wt]) and MS17 (*hemB*). The relative amounts of transcript (RAT) are expressed as percentages of the quantities at the time of rifampin addition. Regression lines (dashed lines) are based on mean values from at least two independent experiments. mRNA half-lives were read at a RAT of 50.

not reflect any particular growth phase of the parent strain or the absence or predominance of one single regulatory element. Surprisingly, an astonishing overall reduction of transcription happened upon exit from exponential phase, which applied to the virulence factors *hla* and *spa* as well. Deviations were found for all analyzed global regulators and can be classified into three groups: (i) no transcription, as observed for *agr*; (ii) transcripts that were only present until cells entered post-exponential phase (during this time they were either up-regulated [*arl*, *sae*, *sarA*, *sarB*, *sigB*, and *srr*], down-regulated [*rot*, *sarR*, *sarS*, and *svrA*], or expressed as in the parent strain [*yyc*]), and (iii) transcription throughout the observed time course, as seen for *sarC*.

The complete absence during 15 h of both the *agr* effector molecule RNAIII (29, 50) and RNAII, encoding a quorum-sensing two-component system (37), is supposed to have an influence on the expression of several genes in the *hemB* mutant. It could explain the lack of *sae* transcription after the exponential phase in the *hemB* mutant, since *sae* transcription requires *agr* in post-exponential phase (24, 49). Although *agr* is the only known inhibitor of *fnbA*, it was not (yet) expressed in either strain when *fnbA* transcription decreased (Fig. 2 and 7). In addition, in an RNAIII-defective 8325-4 background, up-regulation of *fnbA* transcription in the corresponding *hemB* mutant was still observable (65). These findings support the idea that some additional repressor must exist (68).

sar transcription had been shown to be reduced in an 8325-4 *hemB* mutant, suggesting that the observed increased *fnbA* levels were not connected to *sar* but that other regulators were likely responsible for the observed phenomenon (65). By using Northern blot techniques and by monitoring the entire growth cycle, we obtained a more detailed image. We were able to show that in the *hemB* mutant, *sarC* was the only regulator transcript present throughout growth and that, in consequence, its exclusive and required activator σ^B was present and active as well. The absence of σ^B in the *hemB sigB* double mutant dominated over residual *sar* expression, as *fnbA* transcription ceased in that strain as well.

Since findings of reduced *sar*, but increased *fnbA* and *clfA*, transcription in an 8325-4 *hemB* mutant stem from just one time point of late log phase (65), comparison with our results is difficult. By evaluating mRNA levels in MB79 (2 and 3 h) and MS17 (3 and 5 h) during late exponential phase, we found increased *fnbA* transcription in the Newman *hemB* background. However, overall *sar* mRNA levels were increased, while *clfA* seemed to be lower in late log phase (Fig. 7). Apart from sampling differences, the diverse genetic backgrounds might have had an influence on relative expression levels as well, as seen for *agr*.

While transcription of most of the analyzed genes was concentrated to the exponential phase in the *hemB* mutant, some of the gene products were presumably present and active until stationary phase, as was found for σ^B. Results for *fnbA* and *isaA* transcription presented here combined with data reported for FnbA and IsaA strongly suggest that these two proteins persist as well (34, 65). Speculating that the energy-restricted *hemB* mutant does not express needless genes, the increased transcription levels of *arl*, *sae*, *sar*, and *srr* in exponential phase suggest that these regulators are involved in the control of its transcriptome. A subject of future work is to determine the presence and activity of the aforementioned regulators in vitro as well as in vivo. For this purpose, reporter systems have to be used with care, as seen for luciferase, which otherwise reliably detects fluctuating gene expression. Apparently, in the *hemB* mutant, conditions are such that the rather unstable luciferase (half-life of 2 h) (28) is hardly translated, rapidly degraded, or inactivated. Availability of ATP within the *hemB* mutant could be ruled out as the reason for reduced luciferase activity, as luciferase measurements were performed with cell extracts in excess of ATP. Data indicate that the used reporter system poorly reflects σ^B activity in wild-type stationary-phase bacteria as well. Whether some similarity between their physiological state and that encountered with the *hemB* mutant exists remains to be investigated.

Interestingly, both *spa* and *hla* transcription started earlier in the mutant (Fig. 4). This was also observed for *rot*, *asp23*, and 2.9-kb *clfA* transcription (Fig. 4, 6, and 7), arguing against the idea that SCVs are cells stalled in early growth phase, as suggested by the absence of *agr* and exoprotein production and the prolonged transcription of genes typically expressed in early growth phase, like *fnbA* and *isaA* (Fig. 2, 5, and 7). Whether the almost-uniform stop of transcriptional activity upon exit of the exponential phase is a coincidence or correlates with a yet uncharacterized transition process remains open. A link to reduced ATP levels seems difficult to make, since the reduced ATP levels are rather constant in a COL *hemB* mutant (34). Various metabolic enzymes have been reported to be transcribed after the exponential phase (34), suggesting that transcription focuses on selected and required genes.

Both the premature transcription and the drastic reduction of transcription observed in the *hemB* mutant, for σ^B-dependent and -independent loci, indicate that global alterations of gene expression do happen. Thus, the extraordinary cellular state caused by the interrupted electron transport in *hemB* mutants is linked to altered activity of global regulators and expression of virulence factors.

Long-term follow-up of σ^B-dependent transcription revealed that σ^B was active, with fluctuations, until stationary phase in the parent strain, possibly in response to general changes in growth conditions. With the exception of the *sarC* transcript having its maximal level in post-exponential phase and thereafter declining (Fig. 7), σ^B-dependent mRNA patterns were similar and increased towards stationary phase. This variation in expression of known σ^B-dependent genes was much more pronounced in the Newman *hemB* mutant. While *sarC* and the 2.9-kb *clfA* and 1.5-kb *asp23* transcripts were detectable throughout growth, this was not the case for the 1.6-kb P3-*sigB* and 0.7-kb *asp23* transcripts.

A recent study of clinical thymidine-auxotrophic SCVs concluded from pigmentation and *sarC* and *asp23* transcriptional patterns that σ^B has a lower activity in most of these mutants than in their parents (33). While generally reduced *agr* and *hla* levels were found as well, *spa* levels were frequently higher. The diversity of mRNA levels and expression patterns found for the wild-type strains indicates that these clinical isolates have varying genetic backgrounds. The fact that supplementation of the SCVs with thymidine did not always fully restore the wild-type phenotype furthermore suggests that additional unknown alterations, possibly affecting σ^B regulation, had oc-

curred before or during SCV formation (33). Comparison with our data is further complicated by the fact that the analyzed thymidine-dependent SCVs displayed two classes of colony morphology, the genetic reason for which is yet unknown. Lack of pigment formation is not a reliable indicator of the absence of σ^B activity, since many more factors can influence this trait, e.g., mutation of the synthesizing enzymes or regulators or a defective electron transport (6, 32, 36, 40). However, as judged from the presence of the σ^B-requiring *sarC* or *asp23* mRNA levels, apparently reduced σ^B activity could be observed for several of the analyzed SCVs. The partial inconsistency of these two σ^B-activity indicators further hints at the presence of promoter-specific σ^B modulators and makes it difficult to conclude the relative amounts of σ^B activity from selected mRNA levels.

Based on these observations, we postulate the presence of factors modulating σ^B activity in the recognition of its promoter consensus sequences under certain circumstances. Such σ^B modulators may also explain diverging results concerning σ^B activities in different genetic backgrounds and under various experimental conditions.

Yet, *sigB* was definitely not an essential locus for the in vitro SCV phenotype, as its inactivation did not affect colony morphology, growth, or antibiotic resistance. Nevertheless, σ^B is strictly required for high-level expression of at least two important virulence factors in vitro, FnbA and ClfA. In vivo studies with a guinea pig model of device-related infection of wild-type Newman showed that alternative regulatory pathways seem to be active in vivo, as shown for *coa*, which despite its σ^B-dependent consensus promoter sequence (7) needs Sae in vivo (25). However, full ClfA expression still requires σ^B, the only *clfA* regulator identified so far (25). The general importance of σ^B for virulence is a continuing field of investigation. In several animal models, no permanent difference between the wild type and *sigB* mutants was observed (18, 27, 47). Analogous to the σ^B-independent adherence observed in vitro (18), stemming from a basal σ^B-independent expression of *fnbA* and *clfA*, and redundant, σ^B-independent, adhering proteins like FnbB and ClfB (7), σ^B-mediated adherence might not be required for all types of infection. Nonetheless, in a mouse model of septic arthritis, lack of σ^B resulted in attenuated infection (30). In that model, *sigB* mutants displayed a reduced ability to survive in the bloodstream and to persist in kidneys and joints, leading to a reduced mortality.

It is reasonable that the expression of FnbA, the more effective adhesion and invasion factor of the two redundant FnBPs FnbA and FnbB (26), together with ClfA, which has been shown to protect bacteria from phagocytosis by macrophages (52), might significantly contribute to persistence in the host, a characteristic feature of SCVs. Whether in vivo FnbA, ClfA, and σ^B indeed play a crucial role in the establishment of SCV infections remains to be determined.

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