

## A Site-Directed *Staphylococcus aureus hemB* Mutant Is a Small-Colony Variant Which Persists Intracellularly

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Although small-colony variants (SCVs) of *Staphylococcus aureus* have been recognized for many years, this phenotype has only recently been related to persistent and recurrent infections. Clinical *S. aureus* SCVs are frequently auxotrophic for menadione or hemin, two compounds involved in the biosynthesis of the electron transport chain elements menaquinone and cytochromes, respectively. While this observation as well as other biochemical characteristics of SCVs suggests a link between electron-transport-defective strains and persistent infections, the strains examined thus far have been genetically undefined SCVs. Therefore, we generated a stable mutant in electron transport by interrupting one of the hemin biosynthetic genes, *hemB*, in *S. aureus* by inserting an *ermB* cassette into *hemB*. We isolated a *hemB* mutant, due to homologous recombination, by growth at a nonpermissive temperature and selection for erythromycin resistance. This mutant showed typical characteristics of clinical SCVs, such as slow growth, decreased pigment formation, low coagulase activity, reduced hemolytic activity, and resistance to aminoglycosides. Additionally, the mutant was able to persist within cultured endothelial cells due to decreased alpha-toxin production. Northern and Western blot analyses showed that expression of alpha-toxin and that of protein A were markedly reduced, at both the mRNA and the protein level. The SCV phenotype of the *hemB* mutant was reversed by growth with hemin or by complementation with intact *hemB*. Hence, a defect in the electron transport system allows *S. aureus* SCVs to resist aminoglycosides and persist intracellularly.

*Staphylococcus aureus* is one of the most feared pathogens, causing severe morbidity and often rapidly fatal infections (30, 47). It produces toxins and is resistant to various antibiotics. Most staphylococci are resistant to penicillin, and depending on local epidemiological conditions, a significant number of isolates are resistant to methicillin, clindamycin, erythromycin, aminoglycosides, and/or quinolones (17, 42). However, of patients infected with susceptible organisms, some demonstrate poor clinical and bacteriologic response to standard antimicrobial regimens (32, 47). Even in patients whose acute infection initially seems to respond to antimicrobial treatment, chronic relapsing disease characterized by long periods of quiescence between episodes of acute illness may occur (13, 23). Specific microbiological factors associated with such antibiotic failures have not been fully defined, but small-colony variants (SCVs) of *S. aureus* have been recovered from patients with persistent and relapsing infections (35, 36).

*S. aureus* SCVs were first described >80 years ago, and a number of studies support a pathogenic role for SCVs in diseases (1, 2, 31, 34, 38, 40, 41, 47, 50). Although *S. aureus* SCVs have been recognized for many years, the connection of this phenotype to persistent and recurrent infections has only recently been appreciated (36). SCVs represent subpopulations of *S. aureus* that grow slowly on routine media and yield small, nonpigmented, nonhemolytic colonies (35, 36, 38, 50). Biochemical characterization of the large majority of clinically isolated SCVs suggests specific defects in electron transport (25, 35, 36, 49). Clinical and laboratory-generated *S. aureus*

SCVs are frequently auxotrophic for menadione or hemin, two compounds required in the biosynthesis of the electron transport chain components menaquinone and cytochromes, respectively (1, 3, 4, 25, 35, 36, 46). Recently, electron-transport-deficient *S. aureus* SCVs were found to persist inside cultured endothelial cells (4). These SCVs are phagocytized by, but do not lyse, cultured cells. The intracellular location may shield the SCV from the host immune response and antibiotics. While these observations suggest a link between electron-transport-defective strains and persistent infections, defined mutants are required to provide more definitive evidence for these connections. The strains examined thus far have been genetically undefined SCVs, and the clinical strains exhibit a high rate of reversion to the large-colony form. In addition, genetically undefined strains may carry mutations in more than one virulence factor, especially since the clinical SCVs show multiple phenotypic changes compared to the parent strains (35–37). In order to test the characteristics of SCVs of *S. aureus* and to support the hypothesis that defects in electron transport promote the development of intracellular persistence, we generated a stable mutant in electron transport by interrupting *hemB* in *S. aureus*, which is a biosynthetic gene for hemin biosynthesis (aminolevulinic acid dehydratase). This mutant showed typical characteristics of clinical SCVs and was able to persist within cultured endothelial cells.

### MATERIALS AND METHODS

**Bacterial strains, media, and plasmids.** *S. aureus* 8325-4 (NCTC 8325 cured of prophages; plasmid free) (33) was used to isolate the chromosomal DNA for amplification of the *hemB* gene and to generate the *hemB* mutant. *S. aureus* SA113 (16) is a restriction-negative mutant and was used for transformation. Recombinant DNA molecules prepared in *Escherichia coli* were passaged in SA113. The protein A-negative mutant *S. aureus* DU5875 (29), the *agr*<sup>+</sup> strain *S. aureus* RN6390 (43), and the *agr* mutant strain *S. aureus* RN6911 (43) were taken

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as control strains in Western blot analyses. *E. coli* DH5 $\alpha$  [*supE44*  $\Delta$ *lacU169* ( $\phi$ 80*lacZ* $\Delta$ M15) *hsdR17 recA1 endA1 gvrA96 thi-1 relA1*] (14), *E. coli* GM161 (*dam dcm*; kindly provided by R. Brückner), *E. coli* TG1 [*supE hsd* $\Delta$ 5 *thi* ( $\Delta$ *lac-proAB*) F'(*traD36 proAB<sup>+</sup> lacI<sup>+</sup> lacZ* $\Delta$ M15)] (7), and *Staphylococcus carnosus* TM 300 (9, 11) were used as cloning hosts. *E. coli* RP523 is a *hemB*-defective mutant selected by neomycin resistance (26) and was used to test the ability of the cloned *S. aureus hemB* gene for complementation.

*E. coli* strains were grown in Luria-Bertani medium, whereas *S. aureus* was routinely cultured in Trypticase soy broth (TSB) and Trypticase soy agar (TSA) (39). Chemically defined medium (CDM) (44) was used for growth studies of the *hemB* mutant and to determine the amount of hemin appropriate for supplementing the *hemB* mutant.

pUC19 (51) was used as a cloning vector in *E. coli*. pEC5 is composed of pUC19 and the staphylococcal transposon Tn551 (19), containing the erythromycin cassette *ermB*. Plasmid pBT9, composed of parts of pBR322 and pTV1ts (52), is a shuttle vector able to replicate in *E. coli* and staphylococci with a temperature-sensitive replicon for staphylococci. This vector was used to inactivate *hemB* in the genome of *S. aureus* 8325-4. To enhance *hemB* expression in staphylococci, plasmid pCX19, a derivative of the xylose-inducible expression vector pCX15 (48), was used. Plasmids pCE3, pCE5, pCE8, and pCE12 were constructed in this study.

**DNA manipulations and transformation.** DNA manipulations, plasmid DNA isolation, and transformation of *E. coli* were performed by standard procedures (39). *S. carnosus* was transformed with plasmid DNA by protoplast transformation (10, 11), and *S. aureus* was transformed by electroporation (24). For DNA preparations from *S. aureus*, cell wall was digested with 10 U of lysostaphin/ml (Ambicin L; recombinant; Applied Micro Inc., Tarrytown, N.Y.). The subsequent steps in plasmid preparation are those of the alkaline lysis method established for *E. coli*. Chromosomal DNA from *S. aureus* was prepared as described by Marmur (27). DNA fragments were isolated with the Quiaex DNA gel extraction kit (Qiagen, Hilden, Germany). Selection for resistance to antibiotics in *E. coli* was performed with ampicillin (100  $\mu$ g/ml), erythromycin (200  $\mu$ g/ml), and chloramphenicol (10  $\mu$ g/ml).

PCR was carried out with Vent DNA polymerase (New England Biolabs, Beverly, Mass.). After an initial denaturation step (95°C for 4 min), 25 amplification cycles (95°C for 1 min, 60°C for 2 min, and 73°C for 2 min) were performed, with a final 10-min extension step at 73°C. Primers used to amplify the *hemB* gene were as follows: 5'-CTCGGATCCAACAGCACGGACCATTAATCATATCAACAACC (upper primer, including nucleotides 10 to 43 of the sequence for the *hemB* gene [18]; *Bam*HI restriction site is underlined) and 5'-CTCGGTACCACCTTAATTATCTAAATAGCGACAAATGTCC (lower primer, including nucleotides 1058 to 1088; *Kpn*I restriction site is underlined).

**Construction of a *hemB*-deficient mutant in *S. aureus* by gene replacement.**  
(i) **Cloning of the *hemB* gene.** The *hemB* gene was amplified from chromosomal DNA of *S. aureus* 8325-4 by PCR with primers which contained restriction sites for *Bam*HI and *Kpn*I, yielding a 1,084-bp DNA fragment. The fragment was ligated with pUC19, and *E. coli* DH5 $\alpha$  was transformed with the ligation mixture. Ampicillin, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) were used to select transformants. One representative plasmid was designated pCE3.

(ii) **Insertion of the *ermB* gene.** Plasmid pEC5 was isolated from *E. coli* GM161 and cut with *Bcl*II and *Bam*HI. The 1.4-kb fragment carrying the *ermB* gene was isolated and ligated with the *Bcl*II-linearized pCE3 propagated in *E. coli* GM161. The *Bcl*II restriction site is located at the center (base 481) of the *hemB* sequence. *E. coli* TG1 was transformed with the ligation mixture, and transformants were selected with erythromycin and ampicillin. One representative plasmid was designated pCE5. The *hemB-ermB-hemB* fragment was isolated from pCE5 as a 2.5-kb *Sst*I and *Sal*I fragment, ligated with the temperature-sensitive shuttle vector pBT9, and cloned in *E. coli* TG1. The selection of the transformants was performed with ampicillin. One resulting plasmid which also conferred resistance to erythromycin and chloramphenicol was designated pCE8.

(iii) **Insertional inactivation of *hemB* in *S. aureus* 8325-4.** Since the efficiency of transformation of *S. aureus* 8325-4 with DNA from *E. coli* is very low because of its restriction-modification system, pCE8 was propagated in strain SA113 and then transferred to *S. aureus* 8325-4 by electroporation. Transformants were selected with chloramphenicol (10  $\mu$ g/ml); the transformation efficiency was 10<sup>4</sup> transformants/ $\mu$ g of DNA.

For gene disruption experiments, one transformant was cultivated in TSB with chloramphenicol at 30°C overnight. The culture was diluted into 500 ml of fresh medium containing erythromycin (2.5  $\mu$ g/ml) and grown at 42°C to an optical density at 578 nm (OD<sub>578</sub>) of about 3. Appropriate dilutions were plated onto TSA plates containing erythromycin (2.5  $\mu$ g/ml) and grown at 42°C. Successful transfer of the *ermB* cassette into the *S. aureus* chromosome is indicated by the appearance of colonies that are chloramphenicol sensitive and erythromycin resistant. The successful recombination was verified by PCR; the PCR product of the mutant was 1.4 kb larger than the product of the wild-type strain *S. aureus* 8325-4.

**Hemin supplementation.** In order to determine the amount of hemin appropriate for supplementing the *hemB* mutant, different concentrations of hemin (1, 2, 3, and 4  $\mu$ g/ml) were tested. For this, the mutant was inoculated with 0.05 OD<sub>578</sub> units and was shaken at 150 rpm at 37°C. The different concentrations were tested in a complex medium (TSB) as well as in a chemically defined

medium (CDM). The growth of the strains was determined by measuring the OD in a 2-h interval.

**Coagulase production.** The tube coagulase test was performed by the standard procedure except that the assay was extended for 22 h.

**Hemolysis assay.** The hemolytic activity of the strains was determined spectrophotometrically by measurement of hemoglobin released from rabbit erythrocytes according to the method of Balwit et al. (4). Testing was done on bacteria grown overnight in TSB to late logarithmic phase and pelleted by centrifugation (1,200  $\times$  g for 5 min). The mutant was grown in the presence (1  $\mu$ g/ml) and absence of hemin; the plasmid-complemented mutant was grown in the presence (0.5%) and absence of xylose. The results shown are the means of two experiments (Table 1).

**Biochemical characteristics.** In order to test the biochemical profile of the mutant in comparison to those of the wild-type strain and the plasmid-complemented mutant, we tested the strains by using the Api-Staph system (19 tests; BioMérieux, Marcy-l'Étoile, France), ATB32 Staph (26 tests; BioMérieux), and conventional biochemicals according to the method of Kloos et al. (20). The mutant was tested with and without addition of hemin (1  $\mu$ g/ml), and the plasmid-complemented mutant was tested with (0.5%) and without xylose.

**Susceptibility towards aminoglycosides.** The MICs of gentamicin and kanamycin were determined by the microdilution method, but results were read after 48 h of incubation at 36°C.

**Protein preparation, SDS-PAGE, and Western blot analysis.** In order to detect alpha-toxin production, extracellular proteins were obtained by ethanol precipitation of the culture supernatant. Strains were grown in TSB until the stationary growth phase. After removal of the cells by centrifugation, 1 volume of ethanol (100%, -20°C) was added to the supernatant and incubated at 4°C for 4 to 6 h to allow protein precipitation. Precipitated proteins were centrifuged (15,000  $\times$  g for 30 min), and the pellet was resuspended in H<sub>2</sub>O. The protein content of the preparations was determined according to the method of Bradford (5). Since the amounts of extracellular proteins were markedly reduced in the *hemB* mutant, extracellular proteins of the mutant were concentrated eightfold in comparison to the wild-type strain. The same amounts of proteins for each preparation were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% separation gel, 4% stacking gel). Proteins were stained with Coomassie brilliant blue R-250 (0.1%; Serva, Heidelberg, Germany).

In order to detect protein A, lysostaphin cell lysates were prepared. Strains were grown overnight in TSB, centrifuged, and washed once in Tris-buffered saline (TBS), and the cell pellet was weighed and resuspended in TBS. Lysostaphin (300  $\mu$ g/g of bacteria; Applied Micro; Ambicin L; recombinant) and protease inhibitors (28) were added, and the suspension was incubated for 4 h at 37°C. After centrifugation of the cell debris (25,500  $\times$  g for 45 min), the protein content of the supernatant was measured according to the method of Bradford. The same amounts of proteins for each preparation were subjected to SDS-PAGE.

For Western blot analysis, extracellular proteins or proteins prepared by lysostaphin lysis were separated by SDS-PAGE as described above and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Feldbach, Switzerland) with a semidry blot apparatus. Detection of alpha-toxin and protein A was performed by Western blot analysis with the alpha-toxin antiserum (Sigma, St. Louis, Mo.) diluted 1:30,000 in TBS-Tween 20-normal goat serum (NGS) or the anti-protein A antiserum (Sigma), diluted 1:10,000 in TBS-Tween 20-NGS, respectively. Bound antibodies were detected colorimetrically with anti-rabbit immunoglobulin G (Dako, Hamburg, Germany) or anti-mouse immunoglobulin G (Sigma) alkaline phosphatase conjugates diluted 1:500 in TBS-Tween 20-NGS.

**N-terminal sequencing.** For protein sequencing, the proteins prepared by lysostaphin lysis were separated by SDS-PAGE as described above and blotted onto a polyvinylidene difluoride membrane. After staining with Coomassie blue, a 38-kDa protein band of the xylose-induced pCE12-complemented mutant was cut out and the N-terminal amino acid sequence was determined on an Applied Biosystems 494 gas-phase protein sequencer.

**Northern blot analysis.** Bacterial cells from an overnight culture were adjusted to an OD<sub>578</sub> of 0.05 in 25 ml of TSB and grown as described for Fig. 1. Bacteria (10<sup>10</sup> cells) were pelleted and lysed in 1 ml of TRIzol reagent (Gibco BRL, Berlin, Germany) with 0.5 ml of zirconia-silica beads (0.1-mm diameter) in a high-speed homogenizer (Savant Instrument, Farmingdale, N.Y.). The RNA was isolated as described elsewhere (6). The same amount of total RNA for each strain (10  $\mu$ g), was electrophoresed through a 1% agarose-0.66 M formaldehyde gel and blotted by alkaline transfer (Turbo blotter; Schleicher and Schuell) onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). High-stringency hybridization was performed according to the instructions of the manufacturer of the digoxigenin labeling and detection kit (Boehringer Mannheim), and signals were detected by chemiluminescence. PCR was carried out with AmpliTaq Gold (Perkin-Elmer, Roche Molecular Systems, Branchburg, N.J.). The conditions of *spa* amplification were denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min (33 cycles); the conditions of *hla* amplification were essentially the same, except that the annealing temperature was decreased to 47°C. The primers used to generate digoxigenin-labeled probes by PCR labeling (Boehringer Mannheim) were as follows: protein A (*spa*) upstream primer, 5'-AGGTGTAGGTATTGCATCTGT; *spa* downstream primer, 5'-TTTTTAGCTTCTGACAATAGG; alpha-toxin

(*hla*) upstream primer, 5'-AGTCAGCTCAGTAACAAC; *hla* downstream primer, 5'-TCCAATTTGTTGAAGTCCA. Specific PCR fragments were gel purified, and 5 ng of labeled probe per ml was used for hybridization.

**Intracellular persistence assay.** The intracellular persistence assay was performed as described elsewhere (4) with minor modifications. Briefly, aortic bovine endothelial cell monolayers were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% bovine calf serum to confluence ( $2 \times 10^5$  cells) in 24-well tissue culture plates (Nunc, Roskilde, Denmark). Washed bacteria were adjusted to nearly equal numbers for each strain and added to the washed monolayers. The infected monolayers were incubated for 3.5 h at 37°C in 5% CO<sub>2</sub> to allow adhesion and phagocytosis of the bacteria. The monolayers were then washed three times with DMEM and 10% bovine calf serum to remove nonattached organisms, and then 1 ml of medium containing 10 µg of lysostaphin/ml was added, which effectively eliminated extracellular staphylococci as described elsewhere (4). Incubation in the presence of lysostaphin was continued for 20 min, 24 h, and 48 h. At these time points, the monolayers were washed three times with DMEM to remove lysostaphin. Then 1 ml of sterile water was added to disrupt endothelial cells and to release intracellular organisms. Serial dilutions were made in sterile water. The number of intracellular CFU was determined by plating 100-µl aliquots on TSA in duplicate. The detection limit was 10 CFU. The number of intracellular CFU at each time point was determined in duplicate or triplicate: each point represents the mean of three experiments  $\pm$  standard deviation.

## RESULTS

To address questions concerning possible roles of electron transport-defective *S. aureus* in the pathogenesis of staphylococcal infection, it is first necessary to generate stable, defined mutants. Therefore, we generated a stable mutant in electron transport by interrupting *hemB* in *S. aureus*. The *hemB* gene from *S. aureus* 8325-4 genomic DNA was amplified by PCR and cloned into pUC19 in *E. coli*, yielding plasmid pCE3. An *ermB* cassette was inserted into the *hemB* gene and cloned into the shuttle vector pBT9. After homologous recombination, a *hemB* mutant was isolated by growth at a nonpermissive temperature and selection for erythromycin resistance.

**Complementation of the *S. aureus hemB* mutant and *E. coli* RP523.** The PCR-amplified *hemB* gene was cloned in *S. carnosus* with the vector pCX19, which has a xylose-inducible promoter yielding plasmid pCE12. The *S. aureus hemB* mutant and the *hemB* mutant *E. coli* RP523, each forming small colonies, were transformed with pCE12 or pCE3, respectively. Transformation resulted in normal growth in both *S. aureus* and *E. coli*, and curing *E. coli* RP523 of pCE3 restored the small-colony phenotype.

**Characterization of the *hemB* mutant in *S. aureus*.** (i) **Colony morphology.** The *hemB* mutant showed the following characteristics on agar plates (TSA or CDM) after 24 h of incubation at 36°C: slow growth (pinpoint colonies that are >10-fold smaller than those of the parent strain) and decreased pigment formation (whitish colonies versus golden yellow colonies of the parent strain). Scanning electron microscopy showed that the cells of the *hemB* mutant and those of the parent strain had the same size (data not shown).

(ii) **Growth in liquid culture.** Slow growth of the *hemB* mutant was also observed in liquid medium (TSB or CDM). In addition to the modest change in growth rate, the *hemB* mutant reached stationary phase at a 10-fold lower level of total growth in comparison to the wild type (Fig. 1). In order to determine the amount of hemin appropriate for supplementing the *hemB* mutant, several concentrations of hemin per culture broth were tested. The mutant supplemented with hemin at a concentration of 1 µg/ml showed a growth curve very similar to that of the parent strain (Fig. 1). Supplementation with 2 µg/ml and especially with 3 or 4 µg/ml led to an extended lag phase. With 2 to 6 h of delay, the mutant supplemented with more than 1 µg of hemin per ml grew to log phase and finally reached the same end OD<sub>578</sub> as the wild type (data not shown).

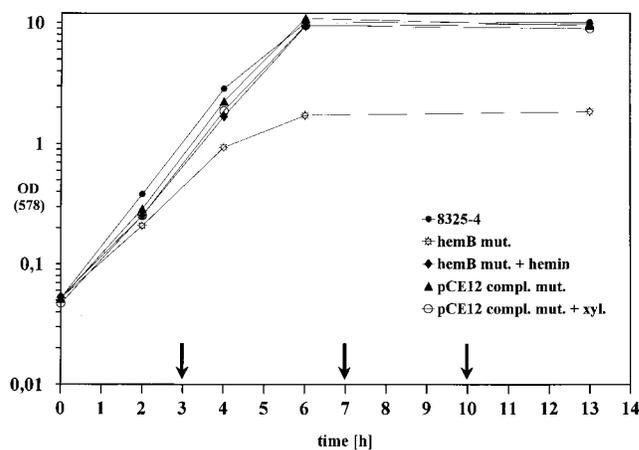


FIG. 1. Growth curves of the parent strain (8325-4), the *hemB* mutant with (1 µg/ml) and without supplementation with hemin, and the pCE12-complemented mutant with (0.5%) and without xylose in TSB. Cells for RNA isolation were harvested during the growth cycle at the indicated time points.

(iii) **Coagulase assay.** In the tube coagulase test, the mutant showed a delayed coagulase reaction, being positive after 22 h of incubation at 37°C whereas the parent strain was positive after 2 h.

(iv) **Hemolysis assay.** The hemolytic activity of the *hemB* mutant was compared to that of the parent strain and the complemented mutant (mutant supplemented with hemin and plasmid-complemented mutant with and without xylose). The *hemB* mutant showed a >90-fold reduction in percentage of lysis of rabbit erythrocytes compared with the parent strain, 8325-4 (0.25 versus 89%) (Table 1). Hemin supplementation restored hemolytic activity. Likewise, the plasmid-complemented mutant with and without xylose revealed the same hemolytic activity as the parent strain, indicating that even in the absence of inducer sufficient HemB is produced.

(v) **Biochemical characteristics.** The biochemical profile of the strains was analyzed in commercial identification test kits and with conventional biochemicals. In contrast to the parent strain and the plasmid-complemented mutant, the *hemB* mutant showed changed biochemical characteristics, such as reduced lactose, turanose, and mannitol fermentation; no nitrate

TABLE 1. Alpha-toxin and protein A production (Western blot analysis) and hemolytic activity of wild-type, *hemB* mutant, pCE12-complemented mutant, and control strains

Strain <sup>a</sup>	Alpha-toxin <sup>b</sup>	Protein A <sup>b</sup>	Hemolytic activity (%) <sup>c</sup>
1	++	++	89
2	-	+/-	0.25
3	++	+	89
4	+	++	89
5	+	++	89
6	++	-	ND
7	+	+	ND
8	-	++	ND

<sup>a</sup> Strains: 1, 8325-4 (wild type); 2, *hemB* mutant; 3, *hemB* mutant supplemented with hemin (1 µg/ml); 4, pCE12-complemented mutant; 5, pCE12-complemented mutant with induction by xylose (0.5%); 6, protein A-negative mutant; 7, *agr*<sup>+</sup> strain; 8, *agr* mutant strain.

<sup>b</sup> ++, strongly positive; +, positive; +/-, weakly positive; -, negative.

<sup>c</sup> Hemolytic activity is shown as the mean of two experiments. ND, not determined.

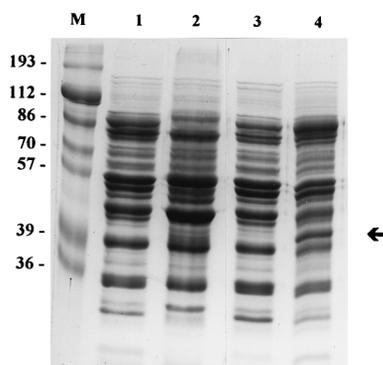


FIG. 2. SDS-PAGE of proteins prepared by lysostaphin lysis. After overnight cultivation in TSB, cells were washed once in TBS, and the cell pellet was weighed and resuspended in TBS. Lysostaphin and protease inhibitors were added, and the suspension was incubated for 4 h at 37°C. After centrifugation of the cell debris, equal amounts of protein were applied to SDS-PAGE gels. Molecular mass markers (in kilodaltons) are indicated on the left. The arrow indicates the position of the postulated HemB protein. Lanes: M, marker proteins; 1, 8325-4 (wild-type strain); 2, *hemB* mutant; 3, pCE12-complemented mutant; 4, pCE12-complemented mutant with induction by xylose (0.5%).

reduction; and reduced *N*-acetylglucosamine utilization. The addition of hemin restored the biochemical profile to that of the parent strain or of the plasmid-complemented mutant with or without xylose.

(vi) **In vitro activity of aminoglycosides.** Regarding antibiotic susceptibility, the mutant was more resistant to the aminoglycosides tested. The MIC of gentamicin was 16-fold higher for the mutant (MIC, 0.5  $\mu\text{g/ml}$ ) than for the wild-type strain (MIC, <0.031  $\mu\text{g/ml}$ ), and the MIC of kanamycin was 8-fold higher for the mutant (MIC, 2.0  $\mu\text{g/ml}$ ) than for the wild-type strain (MIC, 0.25  $\mu\text{g/ml}$ ). When hemin was added to the mutant, MICs identical to those for the parent strain were found. Also, the plasmid-complemented mutant showed MICs identical to those for the parent strain.

(vii) **SDS-PAGE of lysostaphin cell lysates.** The protein band pattern of the lysostaphin cell lysates of the parent strain by SDS-PAGE is very similar to that of the mutant (Fig. 2). The addition of hemin or the presence of pCE12 had no marked influence on the protein band pattern of the mutant. Under *hemB*-inducing conditions, the pCE12-complemented mutant has an additional prominent 38-kDa protein band which is very weak in the absence of xylose and not detectable in the *hemB* mutant with or without hemin. The size of this

protein corresponds to the predicted molecular mass of HemB (36,472 Da, based on the sequence with accession no. S72488).

(viii) **N-terminal sequencing of HemB.** In order to determine whether the additional band of approximately 38 kDa in the pCE12-complemented mutant is HemB, the protein was isolated and its N terminus was sequenced. The obtained sequence (MKFDRHRRLRSSATMRDMVREN) is identical to the predicted HemB N-terminal sequence (18). Thus, the 38-kDa protein represents overproduced HemB.

(ix) **Western blot analysis.** In order to analyze whether the *hemB* mutant produces equal amounts of extracellular and cell-associated proteins in comparison to the wild type, we performed Western blot analysis to detect alpha-toxin and protein A production, respectively. The Western blot analysis showed that alpha-toxin is produced in the parent strain, in the mutant supplemented with hemin (1  $\mu\text{g/ml}$ ), and in the pCE12-complemented mutant independent of the presence or absence of xylose. However, alpha-toxin is not detectable in the *hemB* mutant (Table 1). In control experiments, alpha-toxin was strongly expressed in the *agr*<sup>+</sup> strain (*S. aureus* RN6390) and was not detectable in the *agr* mutant (*S. aureus* RN6911). Production of alpha-toxin by the protein A mutant (*S. aureus* DU5875) was at a level comparable to that in the parent strain, 8325-4.

Protein A was produced in the parent strain as well as in the mutant supplemented with hemin (1  $\mu\text{g/ml}$ ) or complemented by pCE12. However, little protein A was produced in the absence of hemin or the plasmid and was not detectable in the protein A mutant DU5875 (Table 1). As expected, protein A production was much higher in the *agr* mutant than in the *agr*<sup>+</sup> strain.

(x) **Northern blot analysis.** In order to determine whether reduced protein levels correlated with reduced transcription, we performed Northern blot analysis to detect *spa* and *hla* transcripts. For this purpose, samples were taken at the indicated times of the growth curve (Fig. 1). Equal amounts of RNA were isolated and used to determine the amount of *spa* (encoding protein A) and *hla* (encoding alpha-toxin) transcripts in the parent strain, the mutant with and without hemin supplementation, and the pCE12-complemented mutant with and without xylose. The Northern blot analysis of *spa* transcription at different times is shown in Fig. 3A. In the wild type, the amount of *spa* transcript is highest in log phase (sample taken after 3 h of cultivation [Fig. 1]); after 7 h of cultivation, no transcripts were detectable. The noncomplemented *hemB* mutant exhibited no *spa* transcripts. However, if the mutant was

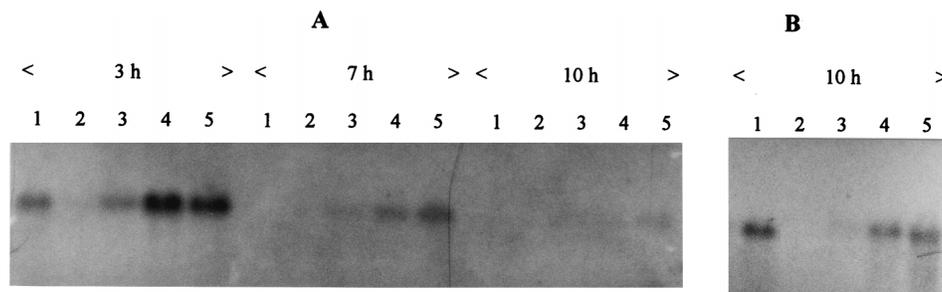


FIG. 3. Northern blot analysis of *spa* (A) and *hla* (B). Isolated RNA was electrophoresed through a 1% agarose-0.66 M formaldehyde gel and blotted by alkaline transfer onto a positively charged nylon membrane. High-stringency hybridization was performed according to the instructions of the manufacturer of the digoxigenin labeling and detection kit, and signals were detected by chemiluminescence. Primers were used to generate digoxigenin-labeled probes by PCR labeling. Specific PCR fragments were gel purified, and a 5-ng/ml-labeled probe was used for hybridization. Transcription of *spa* is shown in the log phase as well as in the early and late stationary phase. Transcription of *hla* is shown in the late stationary phase. *spa* transcripts were determined after 3, 7, and 10 h, and *hla* transcripts were determined after 10 h of cultivation (the 3- and 7-h results are not shown). Lanes: 1, 8325-4 (wild-type strain); 2, *hemB* mutant; 3, *hemB* mutant supplemented with hemin (1  $\mu\text{g/ml}$ ); 4, pCE12-complemented mutant; 5, pCE12-complemented mutant with induction by xylose (0.5%).

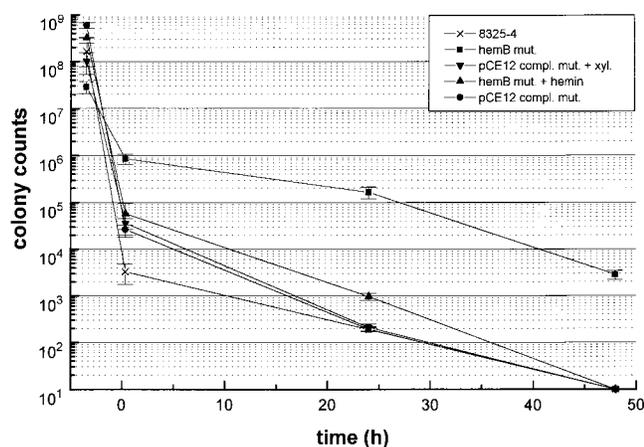


FIG. 4. Intracellular persistence assay within cultured bovine aortic endothelial cells. Aortic bovine endothelial cell monolayers were grown to confluence ( $2 \times 10^5$  cells). Washed bacteria were adjusted to nearly equal numbers for each strain and added to the washed monolayers. The infected monolayers were incubated for 3.5 h at 37°C in 5% CO<sub>2</sub>. The monolayers were then washed, and 1 ml of medium containing 10  $\mu$ g of lysostaphin/ml was added. Incubation in the presence of lysostaphin was continued for 20 min, 24 h, and 48 h. At these time points, the monolayers were washed to remove lysostaphin. Then sterile water was added to disrupt endothelial cells and to release intracellular organisms. Serial dilutions were made, and the number of intracellular CFU was determined in duplicate or triplicate; each point represents the mean of three experiments  $\pm$  standard deviation.

complemented with pCE12 or grown with hemin, *spa* message was detectable after 7 h of cultivation and in the presence of inducer even in late stationary phase (after 10 h of cultivation). Northern blot analysis of *hla* showed that transcription of *hla* was high in early and late stationary phase but not detectable in log phase. Transcription of *hla* in late stationary phase is shown (Fig. 3B). The level of *hla* mRNA was high in the parent strain as well as in the plasmid-complemented mutant with and without xylose. In the noncomplemented mutant, no *hla* transcript was detectable. The addition of hemin resulted in only a low level of *hla* message.

**(xi) Intracellular survival of the *hemB* mutant.** Although hemolytic *S. aureus* strains are readily internalized by bovine endothelial cells, they efficiently lyse the endothelial cells and are thus unlikely to exploit the advantages of this intracellular environment (45). Intracellular persistence assays were performed with the *hemB* mutant to determine whether this mutant could persist intracellularly within aortic bovine endothelial cells more efficiently than the parent strain. Additionally, the pCE12-complemented mutant as well as the hemin-supplemented *hemB* mutant was tested in this assay. In this model of endovascular infection, higher numbers of the mutant were seen following the initial 3.5-h coinubation and a 20-min incubation in the presence of lysostaphin compared to the other strains tested. This is consistent with our previous observation that even after 3.5 h the parent strain caused major disruption of the endothelial cell monolayers (35). Further coinubation in the continuous presence of lysostaphin revealed that >200-fold more *hemB* mutant cells persisted intracellularly after 24 or 48 h of incubation relative to the parent strain and to the plasmid-complemented mutant (Fig. 4). The difference in intracellular persistence was not due to differential susceptibilities of the strains to lysostaphin, which was maintained in the culture medium during the assay to eliminate extracellular organisms. Because hemin supplementation *in vitro* relieved the SCV phenotype of the mutant and enhanced the growth rate, the effect of exogenous hemin on intracellular persistence

was determined for this strain. Addition of hemin (1  $\mu$ g/ml) reduced the numbers of intracellularly surviving CFU of the *hemB* mutant. Thus, supplementation of hemin reduced the intracellular persistence to the level of the parent strain, 8325-4.

## DISCUSSION

*S. aureus* SCVs have been isolated from patients with persistent or antibiotic-resistant infectious diseases (35, 36). The biochemical characterization of these strains suggested specific defects in electron transport (25, 35, 36, 49). Specifically, the following findings are very likely linked to energy-dependent processes: (i) slow growth because cell wall synthesis requires large quantities of ATP, (ii) decreased pigment formation because carotenoid biosynthesis requires electron transport, (iii) resistance to aminoglycosides because their uptake requires the large membrane potential generated by electron transport, and (iv) absence of mannitol fermentation because utilization of mannitol (sugar alcohol) is decreased when electron transport is not used. Additionally, *S. aureus* SCVs, isolated from patients with persistent or antibiotic-resistant infectious diseases, showed auxotrophisms for menadione or hemin, both of which are compounds involved in the biosynthesis of electron transport chain elements (1, 35, 36). While these observations suggest a link between electron-transport-defective strains and persistent infections, our defined *hemB* mutant provides more definitive evidence for these connections.

Heme is the prosthetic group of cytochromes, which play an essential role in electron transport (12). The *hemB* gene is a member of the family of genes encoding enzymes of the porphyrin biosynthetic pathway. This gene codes for the enzyme aminolevulinic acid dehydrase (also called porphobilinogen synthase), which is responsible for the conversion of delta-aminolevulinic acid to porphobilinogen (12).

The stable *hemB* mutant showed typical characteristics of clinical SCVs: (i) slow growth of pinpoint colonies on solid agar, (ii) decreased pigment formation, (iii) reduced hemolytic activity, (iv) decreased coagulase activity, (v) resistance to aminoglycosides, and (vi) changed biochemical characteristics such as reduced lactose, turanose, and mannitol fermentation. The SCV phenotype of the *hemB* mutant was nearly restored by growth with hemin or by complementation with intact *hemB*. The observed delay in growth with higher concentrations of hemin might be due to spontaneous formation of superoxide (4), which may limit the ability of exogenous hemin to completely restore the phenotype. Addition of xylose in order to enhance *hemB* expression showed no major effect. However, a distinct band with a molecular mass of approximately 38 kDa was observed in the protein pattern of lysostaphin lysates. N-terminal protein sequencing showed that this additional protein is HemB.

Mechanisms for the conversion of wild-type strains to SCVs are probably complex. Some of the changes directly relate to interrupted electron transport, e.g., the action of aminoglycosides and some antibiotic cationic peptides is affected due to a reduced electrochemical gradient. At the transcriptional level, we found a decrease in the mRNA for *hla* and *spa*, which indicates that transcription of these genes is affected; probably the two global regulatory systems, *agr* and *sar*, respectively, are involved in this downregulation (15, 22). On the other hand, the export of proteins might be affected, too, because anaerobically grown *S. aureus* cells have a decreased membrane potential and show no alpha-toxin production (4). The observed lack of alpha-toxin production could therefore be due to downregulation and decreased secretion. Some reduction in protein

synthesis could also be due to decreased energy production because protein synthesis is an energy-consuming process.

Since many bacterial species form SCVs (3, 8, 35), the complex phenotypic changes demonstrated by SCVs may prove to be valuable for understanding the relationships between bacterial metabolism and the expression of virulence factors. Formation of *S. aureus* SCVs has important pathogenic implications. Menadione and hemin auxotrophic SCVs have a definite survival advantage because these variants are resistant to aminoglycoside antibiotics (35) or host cationic peptides (21, 37). Recently, it was found that electron-transport-deficient *S. aureus* SCVs persist inside cultured endothelial cells (4), probably because these variants produce very little alpha-toxin. Indeed, previous work using a site-directed mutant of *hla* showed that this alpha-toxin-negative mutant also persisted within endothelial cells (45). The intracellular location may shield the SCVs from host defenses and antibiotics, thus providing one explanation for the difficulty in clearing *S. aureus* SCVs from host tissues. Because intracellular concentrations of hemin are low, the SCV phenotype can be maintained. The intracellular persistence assay with our defined mutant revealed that >200-fold more *hemB* mutant cells persisted intracellularly at 24 or 48 h relative to the parent strain and the plasmid-complemented mutant. Northern blot analysis showed that *hla* mRNA was not detectable in the mutant, whereas *hla* transcription in the parent strain was high.

Hence, the *hemB* mutant is phagocytized by cultured endothelial cells but does not lyse these cells, because the mutant produces very little alpha-toxin. Our *hemB* mutant was also much more resistant to gentamicin compared to the parent strain. Thus, the transformation of *S. aureus* into an SCV may be a potent strategy for protection against host defenses and antibiotic therapy. To further evaluate the persistence and the virulence of the *hemB* mutant, we plan to investigate the mutant in an appropriate animal infection model.

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