Increased Expression of Clumping Factor and Fibronectin-Binding Proteins by *hemB* Mutants of *Staphylococcus aureus* Expressing Small Colony Variant Phenotypes

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Small colony variants (SCVs) of *Staphylococcus aureus* are slow-growing subpopulations that cause persistent and relapsing infections. The altered phenotype of SCV can arise from defects in menadione or hemin biosynthesis, which disrupt the electron transport chain and decrease ATP concentrations. With SCVs, virulence is altered by a decrease in exotoxin production and susceptibility to various antibiotics, allowing their intracellular survival. The expression of bacterial adhesins by SCVs is poorly documented. We tested fibrinogen- and fibronectin-mediated adhesion of a *hemB* mutant of *S. aureus* 8325-4 that is defective for hemin biosynthesis and exhibits a complete SCV phenotype. In this strain, adhesion to fibrinogen and fibronectin was significantly higher than that of its isogenic, normally growing parent and correlated with the increased surface display of these adhesins as assessed by flow cytometry. Real-time quantitative reverse transcription-PCR demonstrated increased expression of *clfA* and *fnb* genes by the *hemB* mutant compared to its isogenic parent. The influence of the *hemB* mutation on altered adhesin expression was confirmed by showing complete restoration of the wild-type adhesive phenotype in the *hemB* mutant, either by complementing with intact *hemB* or by supplementing the growth medium with hemin. Increased surface display of fibrinogen and fibronectin adhesins by the *hemB* mutation occurred independently from *agr*, a major regulatory locus of virulence factors in *S. aureus*. Both *agr*-positive and *agr*-lacking *hemB* mutants were also more efficiently internalized by human embryonic kidney cells than were their isogenic controls, presumably because of increased surface display of their fibronectin adhesins.

During persistent or relapsing staphylococcal infections, a bacterial subpopulation may emerge that can persist for months or even years in a dormant metabolic state (44). These bacteria with decreased metabolic activity are called small colony variants (SCVs) to reflect a phenotypic consequence of their low rate of growth and the formation of minute colonies on solid media. The physiological basis of the SCV phenotype in *Staphylococcus aureus* (46), *Staphylococcus epidermidis*, and other coagulase-negative staphylococcal species has been studied (2, 67). These SCVs frequently were auxotrophic for menadione or hemin, molecules that are required precursors of the components of a complete electron transport chain. Menadione is isoprenylated to form menaquinone, the first electron acceptor in the electron transport chain. The cytochromes that receive electrons from menaquinone and form the chain of higher-redox-potential complexes require a heme prosthetic group. Defects in either menadione or heme biosynthesis that disrupt the functional activity of the electron transport chain lead to an overall decrease in intracellular ATP (3, 44). This metabolic change has pleiotropic effects, such as altering pigmentation, carbohydrate utilization profiles, and exotoxin ex-

pression. In addition, SCVs display increased resistance to some cell wall-active antibiotics and decreased uptake of aminoglycoside antibiotics (45). These differences in susceptibility are presumably due to the decreased capacity for cell wall and teichoic acid biosynthesis and reduction in antibiotic uptake due to decreased membrane potential. One consequence of the observed SCV phenotype is the ability of SCVs to survive within endothelial cells (64) and potentially other cell lines. Intracellular survival of SCVs, perhaps the source of recurrent infections, may be a result of decreased alpha-toxin production of SCVs, which, at wild-type levels, normally lyses eukaryotic cells (57).

Recent studies brought molecular support to this physiological model by showing that a knockout *hemB* mutant, defective for hemin biosynthesis, developed a complete SCV phenotype (66). This was confirmed by restoring the SCV *hemB* mutant into a fully normal phenotype by complementation with an intact *hemB* gene or by growing the auxotrophic SCV mutant in a hemin-supplemented broth medium (66).

As far as we know, the impact of the SCV phenotype on *S. aureus* adhesion to extracellular or host cell components, an important step in the initiation of bacterial infection, has not been described. *S. aureus* and other bacterial species are known to express on their surface protein adhesins (8–11) collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (39). These

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molecules specifically interact with some major plasma or extracellular matrix protein components of normal tissues or those coating biomedical devices (58). The most important host proteins interacting with *S. aureus* are fibronectin (59–61), fibrinogen (18, 20, 32), collagen (39, 41), vitronectin (27), laminin (20, 29), thrombospondin (19), bone sialoprotein (70), elastin (69), and von Willebrand factor (17). Cloning and sequencing of the genes coding for fibronectin-binding proteins FnBPA (53) and FnBPB (23), fibrinogen-binding proteins ClfA (32) and ClfB (35), collagen- and elastin-binding proteins (38, 40, 42), and von Willebrand factor (16) of *S. aureus* have been reported. The two adjacent genes coding for FnBPA and FnBPB are partly redundant since both must be inactivated to eliminate *S. aureus* adhesion to fibronectin (13). The functional significance of each category of *S. aureus* adhesins was explored in vitro and in vivo by using specific mutants. The mutant strains either expressed defective attachment to their respective host proteins or restored wild-type phenotypes by complementation (12–14, 32, 41, 62).

The regulatory circuits governing the expression of MSCRAMMs remain to be fully defined. Available data suggest that transcription of *fnbA* and *fnbB* genes is downregulated by the activation of the *agr* locus (47). The *agr* locus encodes an autoinducing, quorum-sensing, signal transduction circuit that becomes active in the postexponential phase of growth in laboratory cultures (22, 28, 31, 37). The appearance of one *agr* transcript, RNAIII, correlates with downregulation of *fnb*, as well as the *spa* gene encoding protein A (25, 47, 50). However, other MSCRAMM genes like *clfA* appear to be independent of the effect of RNAIII (68). Other regulatory loci, *sar* and/or *sar* homologues (*sarH1*, *sarR*, *sarT*, and *rot*), also play a role in the regulation of MSCRAMMs (30, 34, 52, 56). For example, it is known that SarA, the major regulatory protein encoded within the *sar* locus, can activate *fnb* and *clfA* genes while repressing the transcription of *spa* (5–7, 68).

The aims of this study were to (i) characterize alterations in quantitative adhesion to fibrinogen- and fibronectin-coated surfaces of isogenic *hemB* mutants expressing SCV phenotypes (66) compared to their normally growing parents, (ii) quantify the number of fibrinogen- or fibronectin-binding sites expressed by hemin mutants and their parents, (iii) evaluate the potential impact of the global regulator *agr* activity on the regulation and functional expression of fibrinogen or fibronectin adhesins in wild-type and *agr*-defective mutants of *S. aureus* 8325-4, (iv) evaluate by real-time quantitative reverse transcription (RT)-PCR the expression of *clfA* and *fnb* genes by *hemB* mutants, and (v) correlate the altered expression of fibronectin-binding proteins by *hemB* mutants expressing SCV phenotypes with their endocytosis by human embryonic kidney cells.

MATERIALS AND METHODS

Bacterial strains. Strain 8325-4G is a spontaneous *agr* RNAIII-defective nonhemolytic mutant of strain 8325-4. The defect in RNAIII production of strain 8325-4G was assessed by Northern blot analysis and confirmed by real-time RT-PCR (TaqMan 7770, Perkin-Elmer Applied Biosystems, Foster City, Calif.). The origin of the molecular defect in RNAIII production of strain 8325-4G is unknown, but we could exclude any mutation in either the *sarA* locus (nucleotides 37 to 1317) or *agr* P2-P3 interregion and RNAIII coding region (nucleotides 1480 to 1781) whose DNA sequences were found to be identical to those of strain 8325-4.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype, and/or property	Source or reference
Strains		
S. aureus		
8325-4	NCTC 8325 cured of prophages and plasmids, *rsbU*	www.genome.ou.edu
I10	8325-4 *hemB*::*ermB*, SCV	66
A2	8325-4 *hemB*::*ermB*, pCE12	66
8325-4G	8325-4 defective in *agr* RNAIII production	This study
I15	8325-4G *hemB*::*ermB*, SCV	This study
K1	8325-4 *hemB*::*ermB*, pCE12	This study
Cowan I	NCTC8530, septic arthritis isolate	ATCC 1298
IIb41	Cowan I, *hemB*::*ermB*, SCV	This study
6850	Clinical isolate	ATCC 53657
IIb13	6850, *hemB*::*ermB*, SCV	This study
COL	MRSA strain	www.tigr.org
Ia48	COL, *hemB*::*ermB*, SCV	This study
SA113	8325, restriction deficient	ATCC 35556 (21)
DU5883	8325-4, *fnbA*::*tetM*, *fnbB*::*ermB*	13
DU5880	8325-4, *clfA*::Tn*917*	13
S. epidermidis KH11		43
Plasmids		
pUC19	Cloning vector	71
pCE3	pUC19::*hemB*	66
pBT9	*E. coli-S. aureus* shuttle vector	72
pCE8	pBT9::*hemB*::*ermB*	66
pCE12	pCX19::*hemB*	66

Construction of *hemB*-deficient mutants. *hemB* mutants of *S. aureus* strains 8325-4G, 6850, Cowan I, and COL were constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene, as previously described with wild-type strain 8325-4 and its *hemB* mutant strain I10 (66) (Table 1). In brief, the PCR-amplified *hemB* gene from *S. aureus* 8325-4 genomic DNA was cloned into pUC19, yielding plasmid pCE3 propagated in *Escherichia coli* DH5α. A 1.4-kb *ermB* cassette was inserted into the *hemB* gene and cloned into plasmid pBT9, a shuttle vector conferring chloramphenicol resistance with a temperature-sensitive replicon for staphylococci, yielding plasmid pCE8. After propagation in the restriction-negative strain SA113 of *S. aureus*, pCE8 was transferred to the different strains of *S. aureus* by electroporation and selection of transformants with chloramphenicol (10 μg/ml); the transformation efficiency was 10^4 transformants/μg of plasmid DNA. Disruption of the intact *hemB* gene in the recipient strains was produced by homologous recombination as described previously (66), using growth at a nonpermissive temperature and selection for erythromycin resistance. Successful chromosomal insertion of the *ermB* cassette was indicated by the appearance of chloramphenicol-sensitive and erythromycin-resistant SCV colonies. The successful recombination was verified by PCR; the PCR products of all *hemB* mutants were 1.4 kb larger than the products of their respective isogenic parents.

Complementation of the *hemB* mutants. The PCR-amplified *hemB* gene was cloned in *Staphylococcus carnosus* with the vector pCX19, which has a xylose-inducible promoter, yielding plasmid pCE12 (66). The *hemB* mutants of strains 8325-4 and 8325-4G were transformed with pCE12 as described previously (66). Transformation resulted in normal growth of each *hemB* mutant on both liquid and solid media.

Bacterial adhesion assay. In vitro attachment of *S. aureus* to protein-coated surfaces was measured by the method of Bisognano et al. (4), using polymethylmethacrylate (PMMA) coverslips coated in vitro with purified fibrinogen (32) or fibronectin (13) as described elsewhere. To optimize adsorption of fibronectin from concentrations below 1 μg/ml, PMMA coverslips were precoated with gelatin (1 mg/ml) as described previously (62). Each native or gelatin-surfaced coverslip was coated in duplicate with increasing amounts of immobilized fibrinogen or fibronectin, respectively, by incubation for 60 min at 37°C with three different concentrations (0.25, 0.5, and 1 μg/ml) of either protein in phosphate-buffered saline (PBS), followed by rinsing in PBS as described previously (13, 14). This resulted in a linear coating of PMMA surfaces with fibrinogen and fibronec-

TABLE 2. TaqMan primers and probes used in this study

Gene	Forward primer	Reverse primer	Probe (5′→3′)
fnbB	803F-CACCGAAAACTGTGCAAGCA	889R-TTCCTGTAGTTTCCTTATCAGCAACTT	830T-TAGAAACTTCGCGAGTTGATTTGCCATCG[a]
fnbA	268F-ACAAGTTGAAGTGGCACAGCC	341R-CCGCTACATCTGCTGATCTTGTC	290T-AGAACGGCATCAGAAAGTAAGCCACGTG[a]
clfA	1014F-ATGTGACAGTTGGTATTGACTCTGG	1100R-TAGGCACTGAAAAACCATAATTCAGT	1040T-CGACTGTGTATCCGCACCAAGCAGG[b]
RNAIII	367F-TTCACTGTGTCGATAATCCA	436R-TGATTTCAATGGCACAAGAT	388T-TTTACTAAGTCACCGATTGTTGAAATGA[a]
agrA	34F-CAAAGAGAAAACATGGTTACCATTATTAA	135R-CTCAAGCACCTCATAAGGATTATCAG	83T-AAAAGCCTATGGAAATTGCCCTCGCA[a]
16S	551F-GGCAAGCGTTATCCGGAATT	651R-GTTTCCAATGACCCTCCACG	573T-CCTACGCGCGCTTTACGCCCA[a]
sarA	17F-ACATGGCAATTACAAAAATCAATGAT	167R-TCTTTCTCTTTGTTTTCGCTGATG	45T-CTTTGAGTTGTTATCAATGGT[c]

[a] 5′FAM and 3′TAMRA labeled.
[b] 5′JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) and 3′TAMRA labeled.
[c] 5′FAM Minor Groove Binder and dark quencher labeled.

tin ranging from 41 to 145 ng (28 to 101 ng/cm^2) and 46 to 190 ng (31 to 128 ng/cm^2) per coverslip, respectively.

The adhesion characteristics of parental strains and their *hemB* mutants were evaluated by incubating the protein-coated coverslips with 10^7 CFU of washed cultures of late-logarithmic-phase cells, metabolically radiolabeled with [^{3}H]thymidine during growth without shaking for 5 h at 37°C in Mueller-Hinton broth (MHB) as previously described (4). In some experiments, *hemB* mutants were restored to normally growing cells by supplementing MHB with hemin (1 µg/ml) (66).

Precultures of *hemB* mutants complemented with intact *hemB*$^+$ were prepared by an overnight incubation in MHB supplemented with chloramphenicol (5 µg/ml) to avoid loss of plasmid pCE12. Complemented strains were then incubated for 5 h in antibiotic-free MHB containing both [^{3}H]thymidine as described above and 0.5% xylose to promote *hemB*$^+$ expression. The presence of plasmid pCE12 in the 5-h-radiolabeled cultures was verified by CFU counts on chloramphenicol-containing Mueller-Hinton agar (MHA).

At the end of the attachment period, the fluids containing unbound bacteria were removed, the coverslips were rinsed, and radioactivity on the coverslips was determined as described previously (61). Albumin-coated and gelatin-coated PMMA coverslips were used as controls of nonspecific adhesion to fibrinogen- and fibronectin-coated surfaces, respectively.

Validation of comparative bacterial adhesion data. To validate and normalize bacterial adhesion data of *hemB* mutants compared to their isogenic normally growing parents, whose biomass and cell-associated radioactivity may significantly differ from each other, the following control experiments were performed. First, both CFU and [^{3}H]thymidine cell-associated counts of each SCV or normally growing culture were systematically determined. Average CFU counts of the *hemB* mutant strain I10 represented 38% of those of its isogenic parent strain 8325-4. Similar data were recorded with *hemB* mutants of strains 8325-4G, Cowan, 6850, and COL (data not shown). Complementation of all *hemB* mutants with an intact *hemB* gene led to CFU counts identical to those of their respective wild type parents (data not shown). Average [^{3}H]thymidine cell-associated counts performed in parallel indicated that *hemB* mutants were optimally labeled. Average counts of the *hemB* mutant strain I10 (counts per minute) represented 82% of those of its isogenic parent. Similar data were recorded with *hemB* mutants of strains 8325-4G, Cowan, 6850, and COL (data not shown).

To normalize adhesion data of parental strains and their *hemB* mutants, the data were first scored as the percentage of attached radiolabeled bacteria and then normalized to a fixed inoculum of 10^7 CFU/ml, as previously described (4, 63). Each experiment was performed at least three times, and the results were expressed as means ± standard errors of the means (SEM). The significance of differences in bacterial adhesion of *hemB* mutants compared to their isogenic wild-type or *hemB*$^+$-complemented parents was evaluated by paired *t* tests (49). Statistical significance was evaluated for each individual concentration of fibrinogen or fibronectin immobilized on PMMA coverslips, and data were considered significant when *P* was <0.05 by using two-tailed significance levels.

Quantification of fibrinogen-binding ClfA antigens and FnBPs by flow cytometry. The number of ClfA-mediated fibrinogen-binding sites and FnBP-mediated fibronectin-binding sites displayed by *hemB* mutants of *S. aureus* compared to their isogenic wild-type or *hemB*$^+$-complemented parents was monitored by flow cytometry as previously described (15), except when indicated. The specificity of flow cytometry data for ClfA and FnBPs, respectively, was evaluated by running in parallel ClfA and FnBP knockout mutants of strain 8325-4, respectively.

Late-log-phase cells to be assayed for ClfA after growth (without shaking) for 5 h in MHB were fixed using 0.5% formaldehyde (vol/vol) in PBS (pH 7.2), rinsed in PBS. The bacteria were incubated first for 1 h with purified Fab fragments (1:100) of rabbit anti-ClfA immunoglobulin G antibodies, prepared from whole sera following manufacturer instruction (Immunopure Fab kit; Pierce) and then with (Fab′)$_2$ fragments of goat anti-rabbit immunoglobulin G (1:100) conjugated with fluorescein isothiocyanate (FITC)(Chemicon, Temecula, Calif.). Samples were rinsed twice in FACS buffer (2.5% bovine serum albumin in PBS). The threshold settings for nonspecific fluorescence were obtained by using bacterial cells incubated without primary or secondary antibodies.

Comparative surface display of FnBPs by *hemB* mutants and parental strains was monitored as previously described (12) by incubating formaldehyde-fixed bacteria for 2 h at 4°C with FITC (2 µg/ml)-conjugated fibronectin or FITC-conjugated albumin used as control. Background fluorescence recorded with bacteria incubated with FITC-albumin was subtracted from fluorescence recorded in the presence of FITC-fibronectin (12). Each experiment was performed at least three times, and the results were expressed as means ± SEM. Differences in anti-ClfA or FITC-fibronectin binding by *hemB* mutants compared to their *hemB*$^+$-complemented derivatives or respective parents were evaluated by paired *t* tests (49) and considered significant when *P* was <0.05 by using two-tailed significance levels.

Endocytic uptake assay by human embryonic kidney cells. All media components were from Gibco-BRL. 293 cells (adenovirus type 5 DNA-transformed primary human embryonic kidney) were obtained from ATCC (CRL-1573); maintained in Dulbecco's modified Eagle medium (DMEM)–Nut mix F-12 (Glutamax I) supplemented with 10% fetal calf serum, penicillin (50 IU/ml) and streptomycin (50 µg/ml); and split twice weekly 1:5 by trypsinization. Cells were maintained in humidified air with 5% CO$_2$ at 37°C. The lysostaphin protection assay was performed as previously described (54). Bacterial suspensions were washed with saline, suspended in PBS containing 0.5 mg of bovine serum albumin per ml, and adjusted to 10^7 CFU/ml using the optical density at 540 nm. MHA plates were also inoculated with serial dilutions of these suspensions to control the inoculum. 293 cells (0.2 × 10^6 to 0.3 × 10^6 cells per plate) were transferred to plates at 2 days before the assay and washed with DMEM devoid of antibiotics. Then, cell culture medium was replaced with 0.1 ml of bacterial suspensions, resulting in ~1 × 10^6 CFU/well in invasion medium (DMEM–Nut mix F-12, 1% human serum albumin, 10 mM HEPES), yielding an estimated bacterium/cell ratio (multiplicity of infection) of ~4:1. Cells were incubated for 0, 1, or 2 h at 37°C; bacterial suspensions were replaced by lysostaphin medium (DMEM–Nut mix F-12, 10% fetal calf serum, lysostaphin [20 µg/ml]) and further incubated for 30 min. Cells were subsequently lysed in 1 ml of sterile distilled H$_2$O containing 0.2% Triton X-100. The presence of the detergent does not affect bacterial viability (not shown). Appropriate serial dilutions of the cell lysate in PBS were transferred to plates of MHA, and CFU were counted manually.

Each experiment was performed at least three times, and the results were expressed as means ± SEM. Differences in endocytic uptake of *hemB* mutants compared to their isogenic wild type or *hemB*$^+$-complemented parents were evaluated by paired *t* tests (49) and considered significant when *P* was <0.05 by using two-tailed significance levels.

Real-time RT-PCR. Total RNA was extracted from *S. aureus* strains by using the RNeasy mini Kit (Qiagen). RNA yields were analyzed spectroscopically and by 16S rRNA measurements. mRNA levels were determined using the Platinum quantitative RT-PCR kit (Gibco-BRL). PrimerExpress software (version 1.5; Applied Biosystems) was used to design primers and probes, which were used at 0.2 and 0.1 µM, respectively. All the primers, except the *sarA* probe, a minor groove binder (Applied Biosystems), and probes harboring a 5′FAM (6-carboxyfluorescein) and a 3′TAMRA (6-carboxy-tetramethyl-rhodamine) were synthesized by Eurogentec (Seraing, Belgium) and are listed in Table 2. Fluorescence emission was detected by the sequence detector ABI Prism 7700 and analyzed with Sequence Detector software (version 1.7; Applied Biosystems). The mRNA
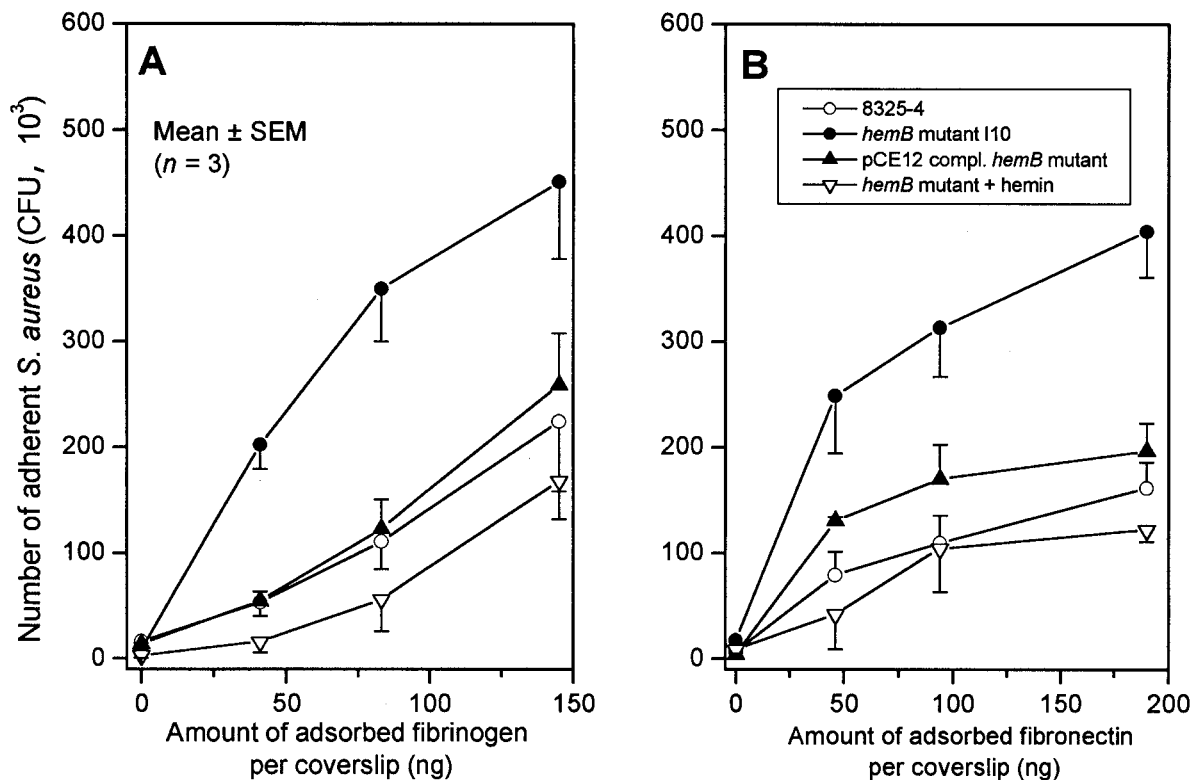
FIG. 1. Adhesion to fibrinogen-coated (A) or fibronectin-coated (B) coverslips of the parental strain (8325-4), its *hemB* mutant (strain I10) with (1 μg/ml) or without supplementation with hemin, and the pCE12-complemented mutant (strain A2) with 0.5% xylose in MHB.

levels of target genes extracted from *hemB* mutants and their isogenic parents were normalized on the basis of their 16S rRNA levels, which were assayed in each round of real-time RT-PCR as internal controls.

RESULTS

Increased adhesion of the *hemB* mutant of *S. aureus* 8325-4 on fibrinogen- and fibronectin-coated surfaces. The bacterial attachment profiles of the parental strain 8325-4 to either fibrinogen- or fibronectin-coated coverslips compared to the *hemB* mutant strain I10, expressed as a function of the amount of adsorbed protein per coverslip, are shown in Fig. 1. The *hemB* mutant displaying an SCV phenotype exhibited a significantly higher attachment to each concentration of immobilized fibrinogen (Fig. 1A) or fibronectin (Fig. 1B), respectively, compared to its normally growing parental strain 8325-4. Average attachment increases of SCV strain I10 over the three coating concentrations of fibrinogen and fibronectin were 3.0- and 2.8-fold, respectively, compared to 8325-4.

Complementation of the *hemB* mutant I10 with plasmid pCE12 expressing the intact *hemB* gene (strain A2), or growth in hemin-supplemented medium, which both led to a normally growing phenotype, reduced bacterial attachment to immobilized fibrinogen or fibronectin to the parental levels (Fig. 1). These data confirmed that the increased attachment of the SCV derivative of 8325-4 to both fibrinogen- and fibronectin-coated surfaces was indeed linked to the mutation in *hemB*.

The *hemB* mutant displayed increased attachment in the

absence of *agr* RNAIII production. To exclude the possibility that the increased bacterial attachment of strain I10 over its parent 8325-4 might simply result from different growth-phase-dependent activities of the global regulator *agr* in the SCV *hemB* mutant compared to its parent, we tested the attachment properties of an isogenic pair of strain 8325-4G, a spontaneous RNAIII-defective mutant of strain 8325-4, and its *hemB* derivative mutant I15. Compared to its *hemB*[+] parent 8325-4G, the RNAIII-defective *hemB* mutant I15 exhibited a significant increase in bacterial attachment, averaging 4.7- and 5.4-fold on the three concentrations of immobilized fibrinogen- (Fig. 2A) or fibronectin-coated coverslips (Fig. 2B), respectively. An unexpected finding was the higher attachment of strain I15 occurring on control coverslips coated with gelatin without any fibronectin (zero point). The higher attachment of strain I15 to gelatin cannot be related to any MSCRAMM thus far described, the 8325-4 family being devoid of the collagen adhesin *cna* gene (39), and may therefore reflect a nonspecific binding mechanism.

Complementation of strain I15 by *hemB*[+] returned it to the parental levels of bacterial attachment to immobilized fibrinogen or fibronectin, as seen in the normally growing strain K1. Taken together, these data ruled out the possibility that upregulation of fibrinogen and fibronectin adhesins in *hemB* mutants displaying an SCV phenotype required the contribution of RNAIII, a major effector of the global regulator *agr*.

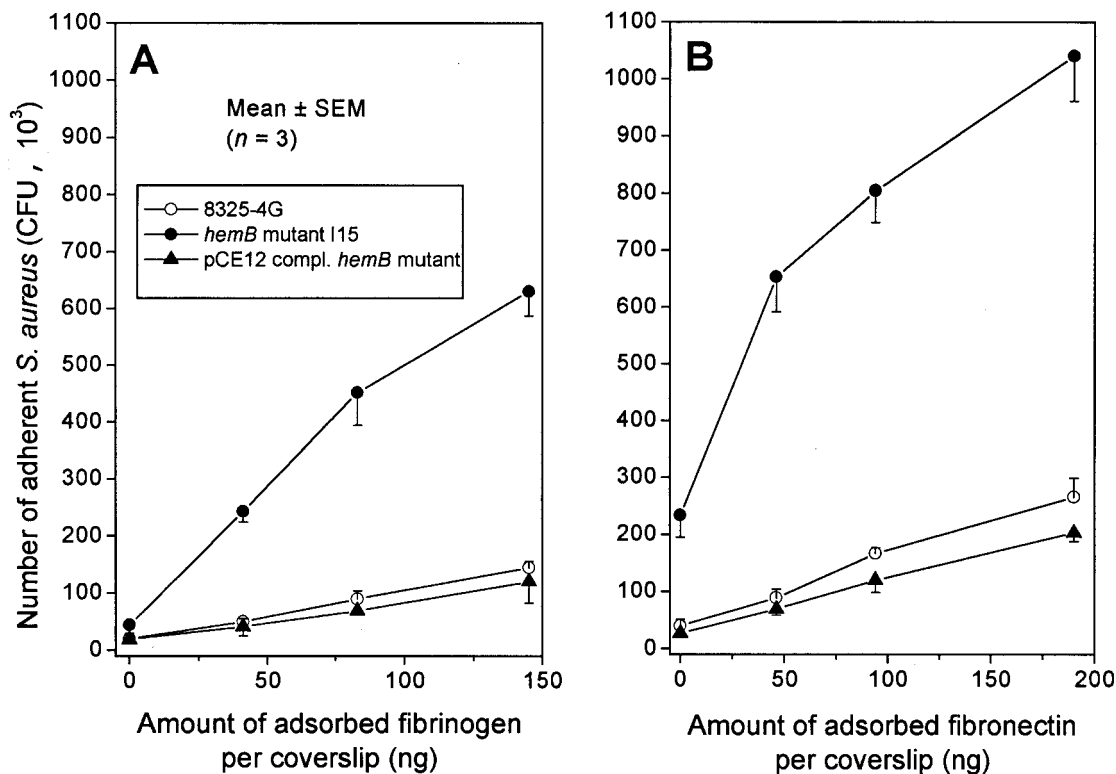**Quantification of fibrinogen-binding ClfA antigens and Fn-**

FIG. 2. Adhesion to fibrinogen-coated (A) or fibronectin-coated (B) coverslips of the parental RNAIII-defective strain (8325-4G), its *hemB* mutant (strain I15), and the pCE12-complemented mutant (strain K1) with 0.5% xylose in MHB.

BPs by flow cytometry. To confirm that increased bacterial adhesion resulted from increased surface display of fibronectin and fibrinogen adhesin molecules by the *hemB* mutants compared to their wild-type or *hemB*⁺-complemented parents, ClfA-mediated fibrinogen-binding sites and FnBP-mediated fibronectin-binding sites were monitored by flow cytometry (15). Figure 3 shows that the *hemB* mutant I10 bound 7.6-fold more anti-ClfA antibodies and 2.1-fold more FITC-labeled fibronectin than its parent 8325-4 ($P < 0.01$). In comparison, the *hemB* mutant I15 bound 6.5-fold more anti-ClfA antibodies and 3.4-fold more FITC-labeled fibronectin than its parent 8325-4G ($P < 0.01$). These differences were indeed linked to the defective *hemB* gene, as they were abolished in the *hemB*⁺-complemented strains A2 and K1. Overall, these flow cytometry data brought further evidence of an increased surface display of fibrinogen-binding *ClfA* and FnBPs adhesins by *hemB* mutants of both strains 8325-4 and 8325-4G, which also explained the higher SCV attachment to surface-bound fibrinogen and fibronectin.

Increased uptake of SCV *hemB* mutants by human embryonic kidney cells. The *hemB* mutant strains I10 and I15 were internalized at an 8.7- and 25.6-fold-higher rate by HEK cells compared to their parents 8325-4 and 8325-4G, respectively, whose uptake was marginal, in a lysostaphin protection assay (Fig. 4). Since recent studies provided evidence that *S. aureus* invasion of 293 cells critically depends on fibronectin bridging between *S. aureus* FnBPs and the host receptor integrin $\alpha_5\beta_1$, the present data strongly suggest that increased surface display of FnBPs by strains I10 and I15 is responsible for increased

internalization of these SCV *hemB* mutants. Restoration of low levels of internalization observed in the *hemB*⁺-complemented strains A2 and K1 by HEK cells, compared to parental strains I10 and I15, respectively, is likely explained by their aforementioned decreased surface display of FnBPs (Fig. 4).

Evaluation of steady-state mRNA of *clfA*, *fnbA*, and *fnbB* genes in *hemB* mutants and parental strains of *S. aureus*. To explore the mechanisms leading to increased expression of fibrinogen and fibronectin adhesins in SCV *hemB* mutants of 8325-4 and 8325-4G compared to their respective parents, their contents in steady-state mRNAs encoded by *clfA*, *fnbA*, and *fnbB* genes were assayed by real-time RT-PCR (TaqMan). To normalize the transcript levels of *clfA*, *fnbA*, and *fnbB* assayed in these differently growing strains, 16S rRNA of both *hemB* mutants and parents were assayed in parallel by real-time RT-PCR. Figure 5A shows significantly increased contents of *clfA*, *fnbA*, and *fnbB* mRNAs in the *hemB* mutant I10, averaging 169% ± 13%, 148% ± 15%, and 148% ± 18%, respectively (means ± SEM), of those assayed in the parent 8325-4.

Despite the fact that the RNAIII-defective strain 8325-4G yielded contents of *clfA*, *fnbA*, and *fnbB* mRNAs which were reduced by approximately one-half compared to those of the *agr*⁺ parent 8325-4, the *hemB* mutant I15 also showed significantly increased levels in mRNAs for each of these adhesins (Fig. 5A). Namely, *clfA*, *fnbA*, and *fnbB* mRNAs assayed in strain I15 in the absence of any significant RNAIII production represented 241% ± 18%, 179% ± 15%, and 189% ± 34%, respectively (means ± SEM), of those assayed in strain 8325-4G.

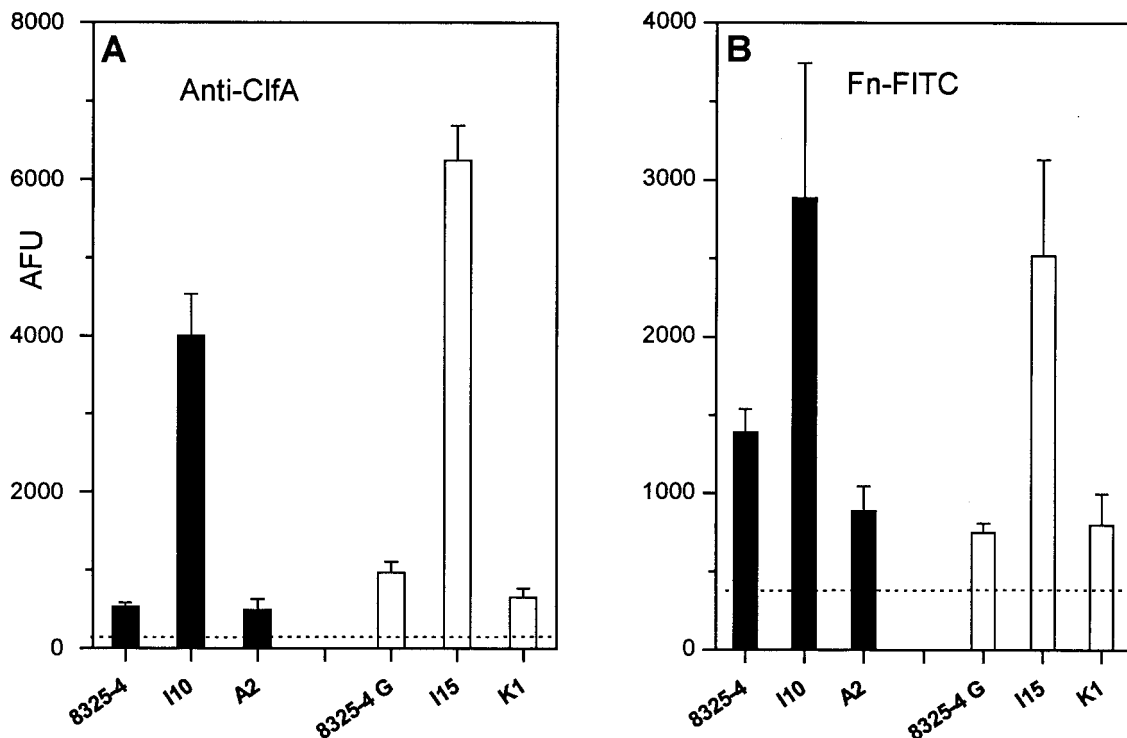To evaluate whether the different levels of *clfA*, *fnbA*, and

FIG. 3. Binding of soluble anti-ClfA antibodies (A) or FITC-labeled fibronectin (Fn-FITC) (B) by strain 8325-4 or its *hemB* (strain I10) or pCE12-complemented (strain A2) *hemB* mutants was compared to that of the RNAIII-defective strain 8325-4G and its respective *hemB* (strain I15) or pCE12-complemented (strain K1) *hemB* mutants. Dashed lines represent the baseline binding of anti-ClfA and Fn-FITC by *clfA* mutant DU5880 (A) and *fnbA fnbB* mutant DU5883 (B) strains, respectively. Results are means + SEM (error bars) of three experiments performed in duplicate and scored in arbitrary fluorescence units (AFU).

fnbB mRNAs assayed in the 8325-4 and 8325-4G parental strains and their respective *hemB* derivatives were due to functional changes in their major global regulators *sar* and *agr*, we assayed in parallel the quantitative levels of mRNAs coding for SarA, RNAII, and RNAIII, respectively. Figure 5B shows that both *hemB* mutants I10 and I15 and the RNAIII-defective parent 8325-4G yielded similar decreased levels of *sarA* transcripts compared to the *agr*[+] parent 8325-4. Thus, these data revealed that the levels of *clfA*, *fnbA*, and *fnbB* messages are not correlated with *sarA* transcript levels in these different strains.

More contrasted results were found when assaying RNA transcript levels originating from P2 compared to P3 *agr* promoters in normally growing versus *hemB* mutant strains (Fig. 5B). In strain 8325-4 and its *hemB* mutant I10, identical levels of AgrA-encoding mRNAs reflecting the P2 promoter activity were recorded. In contrast, RNAII levels in strain 8325-4G and the *hemB* mutant I15 represented only 27 and 67%, respectively, of the levels found in strain 8325-4. Thus, there was no consistent link between RNAII levels of the P2 *agr* promoter and the level of *clfA*, *fnbA*, and *fnbB* messages found in these different strains.

The most impressive differences were observed in the P3-derived RNAIII transcript levels of 8325-4 compared to the three other strains. While the sharp reduction in RNAIII transcript levels compared to the *agr*[+] parent 8325-4 essentially confirmed that both strain 8325-4G and its *hemB* derivative I15
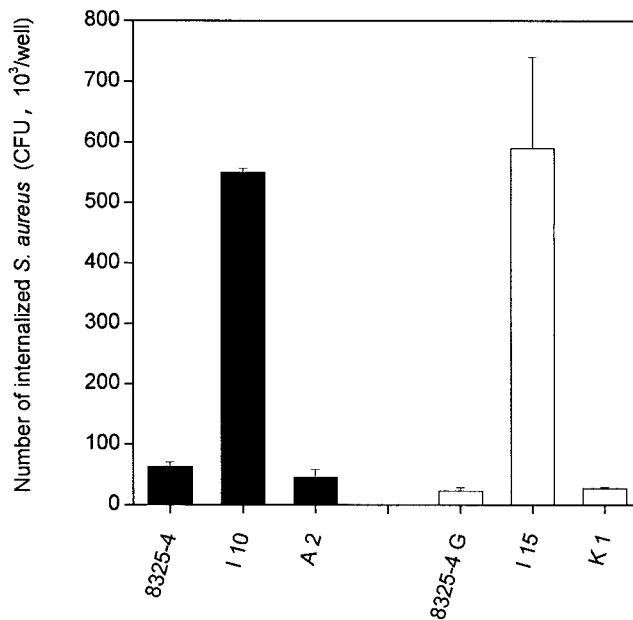

FIG. 4. Increased uptake of SCV *hemB* mutants by human embryonic kidney cells, scored as the number of internalized (lysostaphin-protected) bacteria. Internalization of strain 8325-4 or its *hemB* (strain I10) or pCE12-complemented (strain A2) *hemB* mutants was compared to that of the RNAIII-defective strain 8325-4G and its respective *hemB* (strain I15) or pCE12-complemented (strain K1) *hemB* mutants. Results are presented as means + SEM (error bars)
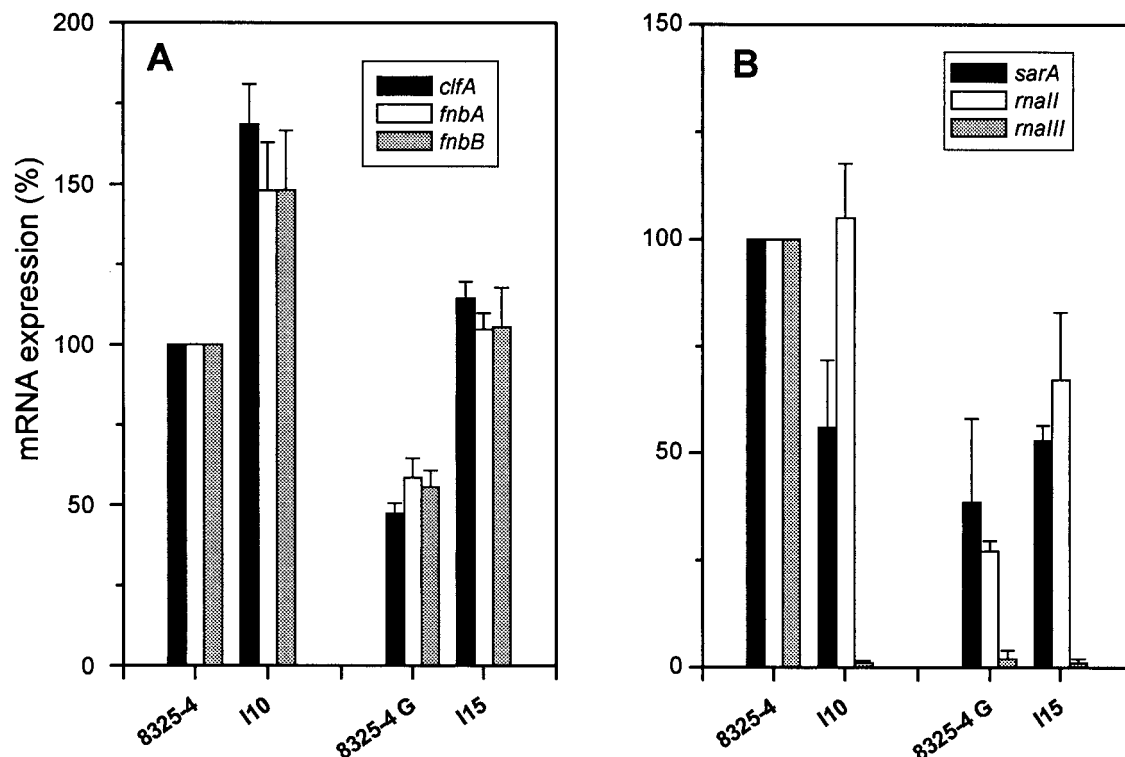
FIG. 5. mRNA expression of *clfA*, *fnbA*, and *fnbB* (A) or *sarA*, *rnaII*, and *rnaIII* (B) genes determined by real-time RT-PCR of 16S rRNA adjusted extracts. Data are presented as means + SEM (error bars) of three experiments performed in triplicate.

displayed stable RNAIII-defective phenotypes, the similar >99% decrease in RNAIII transcript in the *agr*+ *hemB* strain I10 compared to 8325-4 was unexpected. More-extensive studies are required to understand the molecular basis of RNAIII-defective production by the *agr*+ *hemB* mutant strain I10 compared to its isogenic parent 8325-4.

Taken together, neither differences in *sarA* nor *agr* RNAII and RNAIII transcript levels in normally growing parents and their *hemB* mutants could consistently explain the overexpression of *clfA*, *fnbA*, and *fnbB* adhesins by SCVs compared to normally growing organisms. In other words, increased expression of these adhesin genes by *hemB* mutants appear to be mediated by *agr*- and *sar*-independent pathways.

Impact of the *hemB* mutation on adhesin expression by other strains of *S. aureus*. Figure 6 shows flow cytometry data of fibronectin binding by *hemB* mutant strains IIb41, IIb13, and Ia48, compared to their respective normally growing parents Cowan, 6850, and COL. SCV strains IIb41 and IIb13 displayed a significantly higher fibronectin binding of 65% ± 7% and 44% ± 10% (means ± SEM) compared to their isogenic parents Cowan and 6850, respectively. In contrast, the *hemB* mutant strain Ia48 of methicillin-resistant *S. aureus* (MRSA) strain COL exhibited no significant difference in fibronectin binding compared to its normally growing parent. Very low levels of fibronectin binding were exhibited by both strains Ia48 and COL, which were not significantly higher than those of *S. epidermidis* strain KH11, used as a negative control of FnBP display in our assay. The low adhesin content of strain COL and its *hemB* derivative may be due to a previously described
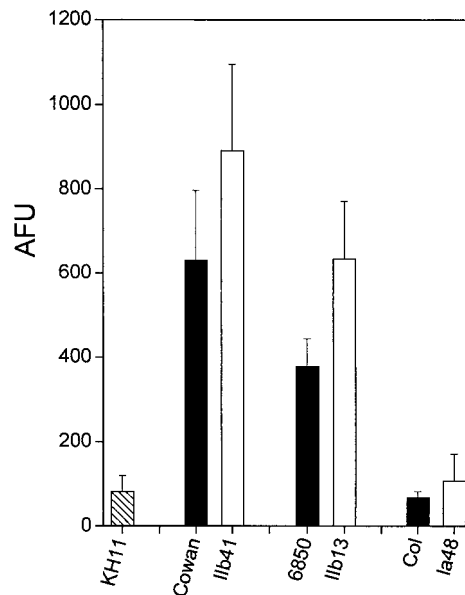

FIG. 6. Binding of soluble FITC-labeled fibronectin (Fn-FITC) by strain Cowan and its *hemB* mutant IIb41, 6850 and its *hemB* mutant IIb13, and MRSA COL and its *hemB* mutant Ia48, compared to *S. epidermidis* strain KH11 taken as a negative control of FnBP display. Results are presented as means + SEM (error bars) of four experiments performed in duplicate and scored in arbitrary fluorescence units (AFU).

mechanism of defective surface display occurring in some clinical and laboratory MRSA isolates (51, 63).

DISCUSSION

Several experimental and microbiological observations provided evidence that a majority of clinical SCV isolates of *S. aureus* are auxotrophic for either hemin or menadione (24, 65). Under natural conditions, any mutation affecting one of the multiple genes of the *hem* operon, namely, *hemAXCDBL*, *hemN*, and *hemYHE* (26), may potentially lead to similar hemin-auxotrophic SCV phenotypes. These considerations also apply to the menadione (3, 65) and thymidine synthetic pathways (24). Extensive genotypic and phenotypic studies of clinical SCVs are hardly feasible because of the difficulty to maintain pure cultures of this phenotype, which generally require addition of antibiotics to protect the slow-growing forms to be overgrown by a minority of normally growing parental organisms (24, 65). The knockout mutation in the *hemB* gene in *S. aureus* represents a very useful experimental system for obtaining a stable SCV phenotype that can be easily reversed by exogenous hemin supplementation or endogenous complementation with an intact *hemB* gene. In three additional unrelated strains, namely, Cowan, 6850, and COL, the inactivation of the *hemB* gene led to pleiotropic changes in bacterial physiology, which essentially mimicked all major features of clinically encountered SCV phenotypes (66). Our data showing increased surface display of both fibrinogen and fibronectin adhesins in *hemB* mutants of strains 8325-4, Cowan, and 6850 stand in contrast with the general notion that SCVs exhibit decreased expression of virulence factors compared to the normally growing parent population of *S. aureus*. While the decreased expression of cytolytic toxins by SCVs may contribute to their prolonged residence and survival in an intracellular milieu without harming the host cells (44), the increased expression of MSCRAMMs by *S. aureus* SCVs might be viewed as an important selective advantage for these slow-growing bacterial cells. In particular, the higher surface display of FnBPs involved in fibronectin-mediated bridging with the host receptor integrin $\alpha_5\beta1$ is known to promote endocytic uptake by some epithelial and endothelial cell lines (54, 55).

Comparison of slow-growing *hemB* mutants with their normally growing parents for quantitative expression of various virulence factors represents a difficult challenge, since major metabolic parameters, such as rates of DNA, RNA, protein, and cell wall synthesis, are significantly decreased in SCVs compared to their normally growing parents. To overcome these potential difficulties, we decided to compare surface display of fibrinogen and fibronectin adhesins by two different procedures, namely, the bacterial adhesion and flow cytometric assays. Both types of binding assays allowed normalization of adhesin expression by equivalent cell numbers of SCVs and wild-type parents and provided convergent evidence for increased surface display of fibrinogen and fibronectin adhesins by slow-growing compared to normally growing populations. While the increased expression of fibronectin adhesins was less important with SCV derivatives of *S. aureus* strains Cowan I and 6850 than with *hemB* mutants of strain 8325-4 and 8325-4G, at least a similar trend was observed with these genetically unrelated strains of *S. aureus*. The defective adhesin expression

of MRSA strain COL and its *hemB* derivative Ia48 is compatible with previous observations showing a defective surface display of major *S. aureus* adhesins in either natural or genetically engineered MRSA strains (51, 63).

An additional difficulty in comparing the regulatory mechanisms of virulence factors of SCVs compared to their normally growing isogenic parents is the unknown influence of growth phase and environmental control on the activities of global regulators of *S. aureus* virulence in the slow-growing populations. In particular, the limiting ATP supply of *hemB* mutants might significantly alter the time course of activation of the global regulators *agr* and *sar* compared to those of normally growing populations (47). To avoid a potentially significant bias in comparing slow-growing with normally growing populations, we also compared the adhesin surface display of one *hemB* mutant with its isogenic RNAIII-defective parent 8325-4G. This spontaneous nonhemolytic mutant whose exact molecular defect has not yet been mapped was preferred to other well-characterized *agr* mutants that already carry erythromycin resistance determinants and also because strain 8325-4G exhibits an extensive defect in RNAIII production. The comparison between strains 8325-4G and I15 provided evidence that the increased display of fibrinogen and fibronectin adhesins could also occur in a *hemB* mutant constructed in RNAIII-defective background and thus ruled out any significant involvement of the growth cycle-dependent global regulator *agr* in the upregulation of these adhesins. Additional support for *agr*- and *sar*-independent mechanisms leading to increased adhesin gene expression and surface display was provided by real-time RT-PCR comparisons, which failed to reveal any consistent link between steady-state transcript levels of each adhesin gene with those of their putative global regulators. While increased transcript levels of *clfA* and both *fnb* genes seem to contribute in part to increased surface display of these important adhesins, the potential contribution of further posttranscriptional and posttranslational regulatory mechanisms should also be considered. This contribution is likely when comparing the mRNA transcript levels with surface expression of ClfA and FnBP adhesins in the *hemB agr* mutant strain I15 and wild-type 8325-4. While adhesion and flow cytometry data showed a markedly enhanced surface expression of ClfA and FnBPs in strain I15 compared to 8325-4, their respective levels of *clfA*, *fnbA*, and *fnbB* mRNA were equivalent. These apparently conflicting data might be explained by a different processing of surface expressed adhesins, as a global consequence of their widely different *agr* activities. A number of studies (see references 1 and 36 for reviews) demonstrated that upregulation of RNAIII promotes the production of serine proteases and their secretion into the extracellular medium. Recent studies documented the contribution of extracellular serine proteases in the transition of an adhesive to a nonadhesive phenotype through rapid degradation of FnBPs and potentially other cell surface proteins (1, 33, 48).

More-elaborate functional genomic approaches evaluating global transcriptomic and proteomic alterations of SCVs compared to their normally growing parents should contribute to identifying the most important regulatory circuits involved in the metabolic adjustments of the slow-growing bacterial populations. The identification and subsequent understanding of the role of these regulatory pathways in vitro and in vivo might

be helpful for the development of new drug categories targeting the persistent slow-growing populations of *S. aureus*.

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