INFECTION AND IMMUNITY, Aug. 2001, p. 4742–4748 Vol. 69, No. 8
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.8.4742–4748.2001

Decreased Amounts of Cell Wall-Associated Protein A and Fibronectin-Binding Proteins in *Staphylococcus aureus sarA* Mutants due to Up-Regulation of Extracellular Proteases

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Received 5 February 2001/Returned for modification 14 March 2001/Accepted 16 May 2001

Data have been presented indicating that *Staphylococcus aureus* cell surface protein can be degraded by extracellular proteases produced by the same bacterium. We have found that in *sarA* mutant cells, which produce high amounts of four major extracellular proteases (staphylococcal serine protease [V8 protease] [SspA], cysteine protease [SspB], aureolysin [metalloprotease] [Aur], and staphopain [Scp]), the levels of cell-bound fibronectin-binding proteins (FnBPs) and protein A were very low compared to those of wild-type cells, in spite of unaltered or increased transcription of the corresponding genes. Cultivation of *sarA* mutant cells in the presence of the global protease inhibitor α_2-macroglobulin resulted in a 16-fold increase in cell-bound FnBPs, indicating that extracellular proteases were responsible for the decreased amounts of FnBPs in *sarA* mutant cells. The protease inhibitor E64 had no effect on the level of FnBPs, indicating that cysteine proteases were not involved. Inactivation of either *ssp* or *aur* in the prototype *S. aureus* strain 8325-4 resulted in a threefold increase in the amount of cell-bound FnBPs. Inactivation of the same protease genes in a *sarA* mutant of 8325-4 resulted in a 10- to 20-fold increase in cell-bound protein A. As the serine protease requires aureolysin to be activated, it can thus be concluded that the serine protease is the most important protease in the release of cell-bound FnBPs and protein A.

Staphylococcus aureus produces several cell surface proteins which bind specifically to different host extracellular matrix proteins and plasma proteins (12, 13, 32). For many of the cell surface proteins a role in colonization and virulence has been demonstrated in animal models of infection (17, 23, 27, 33). Two highly homologous fibronectin-binding proteins (FnBPA and FnBPB), encoded by *fnbA* and *fnbB*, have been characterized (14, 21, 25, 41) and shown to be involved in adherence to damaged heart valves (23) and to promote internalization of *S. aureus* by epithelial cells (9). Although *S. aureus* is primarily considered to be an extracellular pathogen, the intracellular niche could promote long-term colonization and maintenance of chronic infections.

Protein A (Spa), which binds immunoglobulin G (IgG) by the Fc segment, is a major surface protein present in virtually all strains of *S. aureus* (10, 11). Strains of *S. aureus* with a high content of Spa are more resistant to phagocytosis by human neutrophils in vitro than strains with less Spa (34). Reduced virulence of a *spa* mutant compared to that of the corresponding wild type was demonstrated in a mouse intraperitoneal infection (31).

We have recently shown that transcription of the *fnbA* and *fnbB* genes is negatively regulated by *agr* and by an *agr*-independent mechanism that restricts *fnb* mRNA synthesis to the early exponential phase of growth (38). A similar temporal control of *fnb* transcription was also found in another strain of *S. aureus* (Newman) (43). However, only *fnbA* appeared to be regulated by *agr* in this strain. It was also found that *fnbA*, but not *fnbB*, was positively regulated by *sarA*. As for *fnbA* and *fnbB*, transcription of *spa* is negatively regulated by *agr* (20). However, unlike for *fnbA*, transcription of *spa* is negatively controlled by *sarA* (3, 42).

Data from recent studies indicate that both FnBPs and protein A may be degraded by extracellular proteases (3, 26, 42). Four major extracellular proteases are produced by *S. aureus* (1): staphylococcal serine protease (V8 protease) (SspA), a metalloprotease named aureolysin (Aur), a cysteine protease (Scp) named staphopain (18), and a second cysteine protease (SspB) encoded within the same operon as SspA (2, 36). All four proteases appear to be synthesized as preproenzymes, which are proteolytically cleaved to generate the mature enzymes. In the case of the serine protease the proform is enzymatically inactive and needs to be cleaved by aureolysin to become active (8). The proform of SspB that appeared to possess enzyme activity seems to be processed by SspA (36). Which enzymes are involved in the processing of aureolysin and staphopain remains to be determined.

The synthesis of extracellular proteases is positively regulated by *agr* and negatively regulated by *sarA* (2, 20) in such a way that protease production takes place mainly during the postexponential phase of growth, when synthesis of cell surface proteins has ceased. Because of the sensitivity of FnBPs and a limited number of unidentified cell surface proteins to degradation by staphylococcal serine protease, it has been suggested that this enzyme participates in the transition of *S. aureus* cells from an adhesive to an invasive phenotype (26). However, since there are four major proteases which are all regulated in the same way and which are involved in the maturation of each other, we decided to analyze which enzyme(s) is involved in the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source
Strains		
E. coli DH5α	Strain used for propagation of all plasmid constructs	Promega Corp.
S. aureus		
8325-4	Prototype wild-type strain; *rsbU* mutant	30
RN4220	Restriction-deficient mutant of 8325-4	22
DB	Wild-type blood clinic isolate	5
11D2	DB; *sar*::Tn*917* (Em^r)	6
PC1839	8325-4; *sarA*::*km* (Km^r)	2
AK1	8325-4; *aur*::*ermB* (Em^r)	This study
AK10	RN4220; Δ*aur*::*pAK1 (Em^r)*	This study
AK2	8325-4; Δ*ssp*::*ermB* (Em^r)	This study
AK20	RN4220; *ssp*::*pAK2 (Em^r)*	This study
AK3	8325-4; Δ*aur*::*ermB sarA*::*km* (Em^r Km^r)	This study
AK5	8325-4; Δ*ssp*::*ermB sarA*::*km* (Em^r Km^r)	This study
Plasmids		
pKT4	pGEM-T Easy containing *ermB*	42
pAK1	pKT4 containing upstream and downstream *aur* fragments inserted at either side of *ermB*	This study
pAK2	pKT4 containing upstream and downstream *ssp* fragments inserted at either side of *ermB*	This study

[a] Km^r, resistance to kanamycin and neomycin; Em^r, resistance to erythromycin and lincomycin.

degradation of FnBPs and protein A in growing cultures of *S. aureus*. By studying the effects of different protease inhibitors and protease knockout mutants, we have come to the conclusion that staphylococcal serine protease (V8 protease) is the most important for degrading FnBPs and protein A.

MATERIALS AND METHODS

Bacterial strains and plasmids and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were precultured in tryptic soy broth for 16 to 18 h. Cells from 2 ml of preculture were inoculated into 100 ml of brain heart infusion (BHI) broth in a 1-liter baffled flask to an initial optical density at 600 nm (OD_{600}) of 0.25 to 0.3 and were incubated on a rotary shaker (180 rpm) at 37°C. To test the effect of protease inhibitors, parallel overnight cultures of the *sarA* mutant strain 11D2 were transferred to 50 ml of BHI broth (OD_{600} of 0.1) with or without 0.4 U of the universal protease inhibitor α_2-macroglobulin (Boehringer Mannheim) ml^{-1} or 10 μM cysteine protease-specific inhibitor E64 [L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane] (Sigma). Samples of cell lysates and supernatants were taken at indicated time points, and cell-associated FnBPs were analyzed by Western blotting.

Northern blot analysis. Total *S. aureus* RNA was prepared by extraction of lysostaphin-treated cells with hot phenol as described previously (20). Northern blot analysis of *fnb* mRNA and RNAIII, using digoxigenin-labeled antisense RNA probes, was performed as described earlier (38). Serine protease (*sspA*), aureolysin (*aur*), and staphopain (*scp*) mRNAs were analyzed using ^{32}P-labeled probes as described previously (28). DNA fragments of 889 bp (*ssp*), 1,197 bp (*aur*), and 407 bp (*scp*), encompassing the beginning of the structural genes, were generated by PCR using *S. aureus* 8325-4 chromosomal DNA as the template. PCR fragments were radiolabeled to a specific activity of 1×10^8 to 5×10^8 cpm μg^{-1} with [α-^{32}P]dCTP using a random primer labeling kit (Boehringer Mannheim Biochemica). Northern blot images were processed and quantitated using the PhosphorImager 445SI and Image QuanT software (Molecular Dynamics).

Construction of plasmids and protease mutants by allelic replacement. The plasmids were constructed in *Escherichia coli* DH5α, and molecular biology techniques and recombinant DNA manipulations were done as previously described (37). Plasmid DNA was extracted using the Qiagen plasmid mini-kit. *E. coli* transformants were selected on Luria-Bertani (Difco) plates containing 100 μg of ampicillin ml^{-1}.

For construction of strain AK1 (*aur* allelic replacement mutant) two PCR fragments, of 1,064 and 953 bp, encompassing the first 197 and the last 61 codons of the 508-codon aureolysin gene were amplified using forward primers with added *Pst*I and *Eco*RI restriction sites and a reverse primer with added *Spe*I and *Nco*I restriction sites. The PCR fragments were inserted in two steps at either side of the *ermB* cassette in pKT4 to generate plasmid pAK1. The plasmid was then transformed into *S. aureus* RN4220 by electroporation (39). Clones of bacteria were selected on erythromycin plates, and spontaneous chromosomal mutations were eliminated on lincomycin plates (2). The correct insertions were verified by restriction mapping and PCR analysis. Integration of pAK1 into the *aur* gene was verified in one transformant (AK10). Transduction of the *aur* mutation into *S. aureus* 8325-4 using phage Φ11 (29) was performed in order to obtain a double crossover and an allelic replacement of the metalloprotease gene. The *aur* mutation in AK1 was verified by PCR and by Southern blotting using an *aur*-specific probe. The lack of metalloprotease in AK1 was verified by Western blotting (see below).

Strain AK2 (*ssp* allelic replacement mutant) was constructed similarly to AK1. PCR fragments (244 bp [nucleotides 7 to 251] and 332 bp [nucleotides 2693 to 3025], GeneBank accession no. 309515) flanking the *ssp* operon were generated using forward and reverse primers with the same restriction sites as in the construction of pAK1. The PCR fragments were inserted at either side of the *ermB* casette in pKT4 to generate plasmid pAK2, which was then transferred into *S. aureus* RN4220 by electroporation. Recombinants were selected on erythromycin plates and lincomycin plates as described above. Restriction mapping and PCR analysis of one transformant (AK20) using primers internal to the *ssp* operon and flanking primers in the erythromycin cassette confirmed the integration of pAK2 in the *ssp* operon. The *ssp* mutation of AK20 was transduced in *S. aureus* 8325-4 using phage Φ11 (29) to obtain a double crossover and replacement of the *ssp* operon by the *ermB* cassette. Erythromycin-resistant clones were grown on casein agar plates, and colonies with small zones of proteolysis were checked for allelic replacement by PCR and Southern blotting. The loss of SspA in one clone, AK2, was verified by Western blotting (see below).

Strains AK3 (*sarA aur* double mutant) and AK5 (*sarA ssp* double mutant) were constructed by transfer of the *sarA*::*km* mutation from PC1839 into AK1 and AK2 using the transducing phage Φ11 as described before. The mutations were confirmed by PCR.

Western blotting of cell wall proteins and secreted proteins. Cell-associated FnBPs and protein A were released by lysostaphin treatment of equal numbers of bacterial cells (1.2 OD_{600} units) as described previously (20). Released proteins were separated in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels (24) and transferred to polyvinylidene difluoride-based membranes (Immobilon-P; Millipore) using a Bio-Rad Mini Trans-Blot electrophoretic transfer cell as recommended by the supplier. Monoclonal antibodies (goat) against the fibronectin-binding D domains of FnBPA and FnBPB from *S. aureus* (a gift from L. K. Rantamäki, University of Helsinki, Finland) were used and were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibodies (DAKO A/S, Glostrup, Denmark). Protein A was detected by Western ligand blotting using IgG2a from mouse (monoclonal antibody to human CD14; Nordic BioSite Inc.). IgG2a bound to protein A was detected using HRP-conjugated sheep anti-mouse antibodies (Amersham Life Science). For Western blot analysis of the metalloprotease and the serine protease, culture supernatants corre-

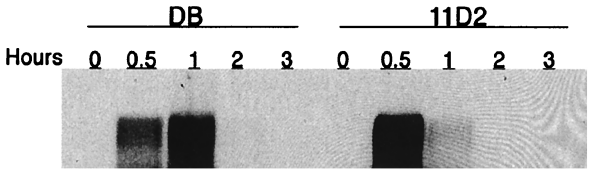

FIG. 1. Northern blot analysis of *fnb* transcripts in *S. aureus* DB (wild type) and the *sarA* mutant 11D2. Equal amounts (10 μg) of total cellular RNA isolated at the indicated time points during growth were analyzed using an RNA probe complementary to conserved regions of *fnbA* and *fnbB*.

sponding to a bacterial density of 0.12 OD_{600} units were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride-based membranes as described above. Polyclonal rabbit anti-metallo-protease and -serine protease antibodies (19) were used and detected with HRP-conjugated sheep anti-rabbit antibodies (Amersham Life Science).

Quantitation of FnBPs and protein A on Western blots was made by the Personal Densitometer SI and Image QuanT software (Molecular Dynamics).

Preliminary sequence data for the *S. aureus* strain COL were obtained from The Institute for Genomic Research website at http://www.tigr.org and for *S. aureus* strain 8325 were obtained from the University of Oklahoma Genome sequencing project website at http://www.genome.ou.edu/staph.html.

RESULTS

Analysis of *fnb* transcripts and cell-associated FnBPs in *S. aureus* DB (wild type) and the *sarA* mutant 11D2. It has been reported that *S. aureus sarA* mutant cells bind less fibronectin than the corresponding parental cells (4, 7, 16, 43). This could be explained at least in part by the recent observation that transcription of *fnbA*, but not *fnbB*, was down-regulated in a *sarA* mutant (43). However, the decreased fibronectin binding in the *sarA* mutant could also be the result of increased production of extracellular proteases (2).

Inactivation of *sarA* in the clinical *S. aureus* strain DB had little effect on the maximum concentration of *fnb* mRNA, although the peak level was reached after 0.5 h (OD = 0.35) in the mutant compared to 1 h (OD = 0.7) in the wild-type strain (Fig. 1). There was no significant difference in growth rate between the strains. It should be pointed out that we used a probe that recognized both *fnbA* and *fnbB* mRNA. In accordance with the transcription analysis, synthesis of cell wall-associated FnBPs was restricted to the first hour of growth in both strains (Fig. 2A). However, in spite of roughly equal levels of *fnb* mRNAs, the amounts of FnBPs were 6- to 10-fold lower in *sarA* mutant cells than in wild-type cells (Fig. 2A). Full-length FnBPs appear on SDS-PAGE as bands with molecular masses of approximately 200 kDa (14, 15, 21). Control experiments using the *fnbA* knockout mutant DU5881 (Fig. 2A), together with previous experiments with the *fnbA fnbB* double mutant DU5883 (38), identified the largest band as FnBPA and the slightly smaller band as FnBPB. The amounts of FnBPA and FnBPB were equally reduced in the *sarA* mutant and the wild-type strain. The intensity of several smaller protein bands previously shown to represent degradation products (38) was also reduced in the *sarA* mutant. As the antiserum is specific for the fibronectin-binding domains of FnBPA and FnBPB, these results are consistent with the reduced binding of fibronectin to *sarA* mutant cells (6, 43).

The slightly altered temporal expression of *fnb* mRNAs in the *sarA* mutant cannot alone explain the reduced level of

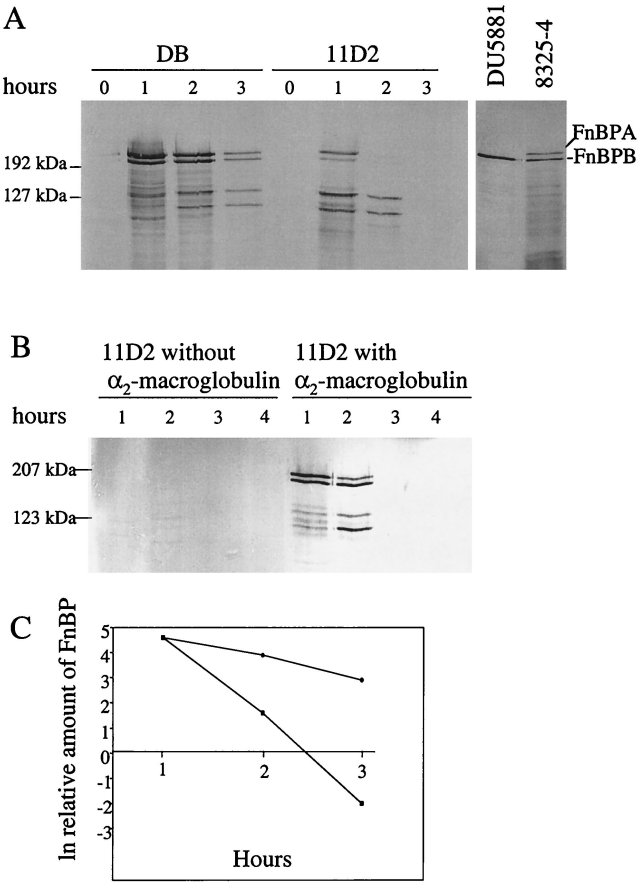

FIG. 2. (A) Cell wall-associated FnBPs in *S. aureus* DB (wild type) and *sarA* mutant 11D2 at different time points during growth. A control experiment with *S. aureus* 8325-4 (wild type) and the *fnbA* knockout mutant DU5881 is shown on the right. (B) *sarA* mutant (11D2) cells cultured with and without 0.4 U of α_2-macroglobulin (protease inhibitor) ml^{-1}. Cell surface proteins from equivalent numbers of cells were separated by SDS-PAGE and analyzed by Western immunoblotting using a monoclonal antibody against the conserved fibronectin-binding domains of FnBPA and FnBPB. Sizes of marker proteins (Kaleidoscope prestained standards) are indicated. (C) Rate of disappearance of FnBPs from cells of strain DB (circles) and 11D2 (squares) during growth. Relative densities of full-length FnBPs from panel A were plotted against time.

FnBPs, suggesting either an impaired translation of the *fnb* transcripts in the *sarA* mutant or an increased release of FnBPs from the mutant cells. Densitometric analysis of Western blots revealed that after de novo synthesis of FnBPs seemed to have stopped (1 h of growth), the concentration of FnBPs per cell decreased about five times faster in the mutant (half-life = 10 min) than in the wild type (half-life = 46 min) (Fig. 2C). As the bacterial growth rate was the same for both strains, this suggests that FnBPs were actively released from mutant cells. However, no significant accumulation of FnBPs or larger degradation products in culture supernatants from *sarA* mutant cells could be demonstrated (data not shown), suggesting that the FnBPs were extensively degraded.

Since the production of proteases is up-regulated in *sarA* mutants (2) and since FnBPs can be degraded by the staphylococcal serine protease SspA (V8) (26), it seems reasonable to

believe that the decreased amount of FnBPs on 11D2 cells was the result of increased protease production. In addition to the V8 protease *S. aureus* produces at least three other extracellular proteases (Aur, Scp, and SspB), which are all up-regulated at the transcriptional level in strain 11D2 (data not shown) and could be responsible for the degradation of FnBPs. To test this, strain 11D2 was cultivated in the presence of α_2-macroglobulin, a universal protease inhibitor that inhibits the activity of all major staphylococcal proteases (26, 35; unpublished results), or the cysteine protease inhibitor E64, which inhibits the staphylococcal cysteine proteases (18, 35, 36). At least 16 times more cell-associated FnBPs were found on cells grown in the presence of α_2-macroglobulin than on cells grown in the absence of inhibitor (Fig. 2B). The addition of the inhibitor had no effect on bacterial growth rate (data not shown). No increase in the amount of FnBPs could be seen when the *sarA* mutant was cultivated in the presence of E64 (data not shown), indicating that cysteine proteases are not involved in the degradation of FnBPs. The amount of FnBPs on *sarA* mutant cells grown in the presence of α_2-macroglobulin was roughly the same as that on wild-type cells grown without protease inhibitor (Fig. 2A), which is in agreement with the roughly equal levels of *fnb* mRNAs in these strains (Fig. 1).

Effect of *ssp* and *aur* knockout mutations on FnBP levels. Our results indicate that serine protease and aureolysin are the major players in the degradation of FnBPs. Specific knockout mutants were therefore constructed. Because of difficulties in genetically manipulating strains DB and 11D2, the mutations were made in the prototype *S. aureus* strain 8325-4, which produces high amounts of proteases and possesses relatively low levels of cell wall-associated FnBPs. The serine protease gene (*sspA*) is part of an operon containing two additional open reading frames, one (*sspB*) coding for cysteine protease and the other (*sspC*) coding for a 12-kDa cytoplasmic protein with unknown function (2, 36). The aureolysin gene, on the other hand, seems to be mono-cistronic. The *ssp* operon and the metalloprotease gene, *aur*, were inactivated by allelic replacement as described in Materials and Methods. Mutants AK1 (*aur*) and AK2 (*ssp*) showed dramatically reduced zones of proteolysis on casein agar plates (Fig. 3). Inactivation of either protease gene resulted in the same (three- to fivefold, in three different experiments) increase in the amount of cell wall-associated FnBPs as for the wild-type strain and a slower disappearance of FnBPs from the cell surface (Fig. 4). Inactivation of *ssp* and *aur* in the *sarA* mutant PC1839 gave similar but less-pronounced results (data not shown). As the serine protease is produced as an inactive proenzyme which is activated through cleavage by the metalloprotease (8), inactivation of *aur* also leads to the loss of serine protease activity. As inhibition of cysteine proteases had no effect on FnBP levels and as the increase in FnBPs was roughly the same in AK1 and AK2, it can be concluded that the serine protease is the most important protease for the degradation of FnBPs.

Effect of serine protease and metalloprotease on protein A. Previous studies in our laboratory have shown that the increased transcription of *spa* (protein A) in the *sarA* mutant PC1839 was not accompanied by a corresponding increase in protein A production (42). To determine whether this was due to degradation of protein A by extracellular proteases, the *aur* and *ssp* knockout mutations were transferred by phage transduction to the *sarA* mutant PC1839 to form strain AK3 (*aur sarA* mutant) and AK5 (*ssp sarA* mutant), respectively. Mutations were verified by PCR using primers specific for the inserted *ermB* gene and the flanking DNA regions. Compared to the parental strain PC1839, AK5 had a slightly smaller zone of proteolysis that was less dense (Fig. 3). Strain AK3, on the other hand, showed no zone of proteolysis. Both mutants showed a 10- to 20-fold increase in cell wall-associated protein A compared to the level in the parental strain PC1839 (Fig. 5). All immunoglobulin-binding bands seen in Fig. 5 represent protein A as they were absent in a *spa* knockout mutant (data not shown). Inactivation of *ssp* or *aur* in strain 8325-4 resulted in comparable increases in protein A (data not shown). Since inactivation of the *ssp* operon (*sspA* and *sspB*) resulted in roughly the same increase in protein A as inactivation of *aur* did, it can be concluded that SspA is the most important enzyme in the degradation of protein A.

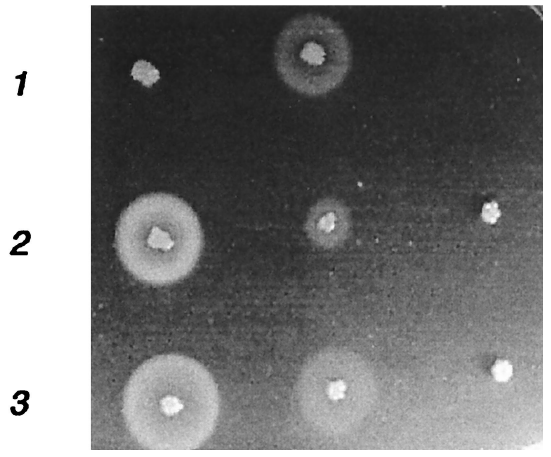

FIG. 3. Zones of proteolysis around *S. aureus* strains grown on a casein agar plate. Row 1, DB (wild type) and 11D2 (*sarA* mutant); row 2, 8325-4 (wild type), AK2 (*ssp* mutant), and AK1 (*aur* mutant); row 3, PC1839 (*sarA* mutant), AK5 (*ssp sarA* double mutant), and AK3 (*aur sarA* double mutant).

Transcription of *ssp* and *hld* (RNAIII) in DB (wild type) and 11D2 (*sarA*). As the serine protease appears to be responsible for the degradation of FnBPs, transcription of *sspA* in *S. aureus* strains DB and 11D2 was analyzed. Northern blot analysis confirmed that transcription of the *ssp* operon was up-regulated in the *sarA* mutant strain 11D2 compared to that in wild-type strain DB (Fig. 6A). In mutant cells the level of *ssp*

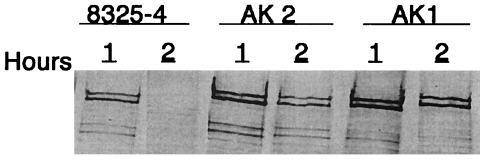

FIG. 4. Cell wall-associated FnBPs in *S. aureus* 8325-4 (wild type), AK2 (*ssp* mutant), and AK1 (*aur* mutant) harvested after 1 and 2 h of growth in BHI broth. Cell surface proteins from equivalent numbers of cells were separated by SDS-PAGE and analyzed by Western immunoblotting using a monoclonal antibody against the conserved fibronectin-binding domains of FnBPA and FnBPB.

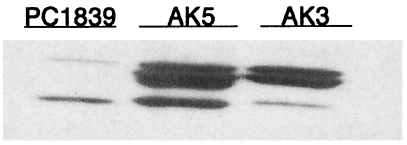

FIG. 5. Cell wall-associated protein A in postexponential-phase (4 h) cells of *S. aureus* PC1839 (*sarA* mutant), AK5 (*ssp sarA* double mutant), and AK3 (*aur sarA* double mutant). Cell surface proteins from equivalent numbers of cells were separated by SDS-PAGE and analyzed by Western ligand blotting using mouse IgG.

transcript increased dramatically during the postexponential phase of growth (2 to 3 h). However, significant amounts of *ssp* transcript were seen at time zero, suggesting that SspA was produced during the early exponential phase of growth at the same time that FnBPs were produced (Fig. 1). Increased protease production by the *sarA* mutant 11D2 was also indicated by large zones of precipitation around bacteria grown on casein agar plates (Fig. 3).

As transcription of *ssp* is stimulated by *agr* (42), levels of RNAIII were also analyzed. Transcription of *ssp* during the early exponential phase of growth of 11D2 was consistent with the presence of RNAIII in cells taken at time zero (Fig. 6B). Although the initial levels of RNAIII were essentially the same in both strains, transcription of *ssp* was severely repressed in the wild-type strain, probably because of high SarA levels. As expected, levels of RNAIII were very low during the mid-exponential phase of growth (1 and 2 h) and increased dramatically during the late exponential and postexponential phases. Notably, the increase in RNAIII appeared later (OD_{600} = 12.0) in the *sarA* mutant than in the wild-type strain (OD_{600} = 8.4), consistent with the expression of RNAIII being positively controlled by *sarA* (16).

DISCUSSION

In the present study we have demonstrated that the decreased expression of FnBPs in 11D2 (*sar* mutant) cells compared to that of the wild-type cells (DB) was due to increased proteolytic degradation of FnBPs rather than to decreased transcription of the *fnb* genes. We also found that the decreased amount of protein A in *sarA* mutant cells was the result of increased protease production. The major protease responsible for the degradation of FnBPs and protein A appeared to be the staphylococcal serine protease (V8).

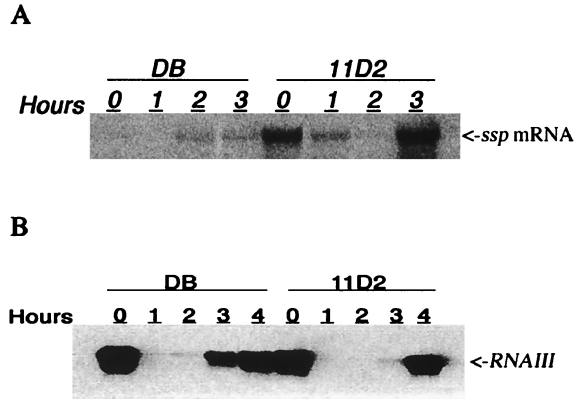

FIG. 6. Northern blot analysis of *ssp* mRNA (A) and RNAIII (B) in *S. aureus* DB (wild type) and the *sarA* mutant 11D2 at different time points during growth.

Contrary to the present results, it was recently shown that transcription of *fnbA*, but not *fnbB*, was down-regulated in a *sarA* mutant (43). In strain Newman, used in a previous study (43), FnBPA appeared to be the dominating FnBP, while in strains DB and 8325-4, which we used, roughly equal amounts of FnBPA and FnBPB were produced (Fig. 2A). In strain 8325-4, FnBPA and FnBPB also seemed to contribute equally to the adherence of *S. aureus* cells to fibronectin-coated surfaces (15). The different relative levels of FnBPA and FnBPB and the different transcription patterns of *fnbA* and *fnbB* with respect to their regulation by *sarA* may be explained by differences between the *fnb* promoter sequences (16, 43). Strain-dependent differences may, however, also be due to variations in the levels of expression of *sarA* and other regulators (e.g., *agr*). Expression of both *fnbA* and *fnbB* was negatively controlled by *agr* in strain 8325-4 (38), while only *fnbA* appeared to be regulated by *agr* in strain Newman (43).

The observation that cultivation of *S. aureus* in the presence of E64 did not significantly affect the amount of cell wall-associated FnBPs strongly suggests that cysteine proteases (SspB and Scp) were not involved in the degradation of FnBPs. The increase in FnBPs seen in the *ssp* knockout mutant was therefore most likely due to the loss of SspA activity. This was supported by the observation that deletion of the metalloprotease that is required for the activation of SspA resulted in the same increase in FnBPs as the *ssp* deletion and by the finding that SspA alone can degrade cell wall-associated FnBPs (26). However, a nonpolar *sspA* mutation in strain SP6391 (derived from 8325-4) did not enhance fibronectin binding, suggesting that the degradation of FnBPs might be the result of the combined activity of two or more proteases (36). However, this discrepancy could also be explained by the observation that binding of soluble fibronectin is not proportional to the amount of cell wall-associated FnBPs. A 16-fold increase in FnBPs resulted in only a twofold increase in fibronectin binding (38). A similar discrepancy between the level of cell-bound FnBPs and binding of soluble fibronectin can also be deduced from data reported previously (43).

FnBPs, protein A, and other cell surface proteins are covalently linked to the cell wall peptidoglycan (40). The release of FnBPs and protein A from the cell wall could therefore also be due to increased autolysin activity. In *sarA* mutants both proteases and autolysin activity are up-regulated, suggesting that the decreased levels of FnBPs and protein A might be the combined result of increased proteolysis and cell wall turnover. However, the increased levels of protein A in the *ssp sarA* and *aur sarA* double mutants compared to that in the *sarA* mutant (Fig. 5) strongly indicate that proteolytic degradation is the most important factor.

Previous reports clearly indicated that the production of FnBPs is inhibited by *agr* and stimulated by *sarA* (38, 43), while the production of proteases is inhibited by *sarA* and stimulated by *agr* (2, 42). This means that the production of serine protease would generally be down-regulated when *fnbA* and *fnbB* are expressed. Under conditions where no extracellular proteases are produced, cell wall-associated FnBPs appeared to be

very stable and disappeared from the bacterial surface at a rate that was proportional to the cell doubling time, meaning that complete down-regulation of the fibronectin-binding phenotype would take several generations. It therefore seems reasonable that FnBPs can be actively released from the bacterial surface soon after de novo synthesis has been turned off. Depending on the regulation of *sarA* and *agr* expression and the relative levels of *sarA* and RNAIII, the amount of FnBPs on the bacterial surface can be modulated to meet the specific requirements during the course of infection.

In the case of protein A the situation seems more complicated, as *sarA* represses transcription of both *spa* and *ssp*. Thus, increased protein A production, as a result of down-regulation of *sarA*, will be counteracted by the concomitant increase in protease production. However, as *agr* is also positively regulated by *sarA* (7), down-regulation of *sarA* might result in a decreased level of RNAIII, which in turn results in decreased protease production and therefore less degradation of protein A. This is consistent with the observation that an *agr sarA* double mutant produced higher amounts of protein A than a *sarA* mutant (42).

In our study we demonstrated that cell wall-associated FnBPs and protein A can be released through the activity of the staphylococcal serine protease. The relevance of this in adaptation to various environmental conditions during the infection process remains to be determined.

ACKNOWLEDGMENTS

We thank Agneta Wahlquist and Lena Norénius for skillful technical assistance. We are also grateful to A. Cheung for providing us with strains DB and 11D2.

This work was supported by grant 4513 from the Swedish Medical Research Council.

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Editor: E. I. Tuomanen