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Variation in Extracellular Protease Production among Clinical Isolates of *Staphylococcus aureus* Due to Different Levels of Expression of the Protease Repressor *sarA*

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***Staphylococcus aureus* produces four major extracellular proteases: staphylococcal serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and staphopain (Scp). Several in vitro studies have suggested that these enzymes are important virulence factors. Here we analyzed the protease production of 92 *S. aureus* strains from infected human soft tissue. Twenty-one strains produced variable zones of proteolysis on casein agar plates, while the remaining 71 strains appeared to be protease negative. The major protease genes were present in all protease-positive ($n = 5$) and protease-negative ($n = 12$) strains analyzed. Northern blotting showed that transcription of the protease genes was suppressed due to increased sigma factor B (SigB)-dependent expression of the protease repressor SarA. Other SigB-dependent traits such as pigmentation and expression of *asp* 23 were also increased in protease-negative compared to protease-positive strains. Inactivation of *sarA* in three protease-negative strains resulted in increased transcription of all protease genes and increased protease production, while overexpression of *sarA* in a strain producing protease at high levels repressed protease production. Our results suggest that the protease genes are conserved among clinical *S. aureus* strains and that the level of SigB-dependent expression of the protease repressor *sarA* determines the level of protease production in each strain.**

Staphylococcus aureus causes a variety of infections, ranging from superficial skin and wounds infections to deep abscesses and septicemia. Recent reports of the U.S. National Nosocomial Infections Surveillance System have ranked *S. aureus* as a leading cause of hospital-acquired bacteremia, pneumonia, and surgical wound infection (11).

The virulence of *S. aureus* is considered to be the result of the coordinated activity of several secreted toxins and digestive enzymes, as well as a large number of proteins on the bacterial surface that bind extracellular matrix and plasma proteins (2, 3, 24, 33). In vitro studies have shown that staphylococcal proteases can cleave and degrade a number of important host proteins, including the heavy chains of all human immunoglobulin classes, plasma proteinase inhibitor, and elastin (42–45), indicating that they are important virulence factors. Recent reports suggest that proteases also play a role in the transition of *S. aureus* cells from an adhesive to an invasive phenotype by degrading bacterial cell surface proteins, such as fibronectin binding protein and protein A (16, 27, 35, 46, 49).

S. aureus produces four major extracellular proteases: serine protease (V8 protease; SspA), a cysteine protease (SspB) encoded within the same operon, metalloprotease (aureolysin; Aur), and a second cysteine protease (Scp; also named staphopain) [2; B. Hofmann, D. Schomburg, and H. J. Hecht, abstract from the 16th Congress of the International Union of Crystallography 1993, Acta Crystallogr. **49**(Suppl.)**:**102, 1993]. All four proteases are secreted as proenzymes, which are proteolytically cleaved to generate the mature enzymes. The proenzyme form of the serine protease is enzymatically inactive and needs to be cleaved by aureolysin to become active (18). In the case of SspB, which is processed by SspA, the proenzyme form appears to be enzymatically active (47). However, which enzymes are involved in the processing of aureolysin and staphopain remains to be determined.

Synthesis of extracellular proteases is activated by *agr* (7, 26, 32) and repressed by *sarA* (14, 32) in such a way that the production of proteases takes place mainly during the late exponential and postexponential phases of growth.

We have observed that the level of protease production varies considerably among clinical isolates of *S. aureus*. To investigate the background for this variation, 92 fresh clinical isolates were analyzed for protease production on casein agar plates. Strains producing different amounts of protease were tested for the presence of protease genes by PCR, and the levels of expression of four major protease genes and of the protease regulators *agr* (RNAIII; activator) and *sarA* (repressor) were analyzed by Northern blotting. Our results suggest that the variation in protease production between strains of *S. aureus* depends on different levels of SigB-dependent *sarA* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. Ninety-two *S. aureus* strains from pyogenic soft tissue infections, isolated at Karolinska Hospital Clinical Microbiology Laboratory in Stockholm, Sweden, were collected at two different time points (1999 and 2000). Strains of *S. aureus* were identified by colony morphology, Gram staining, DNase, coagulase, and the MONOSTAPH slide test (BIONOR, Skien, Norway). Other bacterial strains and plasmids used in this study are listed in Table 1. Screening for protease production was carried out on casein agar plates (8). *S. aureus* strains were precultured in tryptic soy broth for 16 to 18 h. Cells from precultures were inoculated in 100 ml of brain heart

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TABLE 1. Bacterial stains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli DH5α	*E. coli* strain used for propagation of all plasmid constructs	Promega Corp.
S. aureus		
8325-4	Prototype "wild-type" strain; *rsbU* mutant	40
RN 4220	Restriction-deficient mutant of 8325-4	29
PC1839	8325-4 *sarA*::*km* (Km^r)	14
KT111	8325-4 with *ssp* promoter element from 8325-4, fused to *lacZ*, inserted in *geh* (Em^r)	50
CYL316	RN4220(pYL112Δ19)	31
NA1	Clinical strain KS7 *sarA*::*km* (Km^r)	This study
NA2	Clinical strain KS30 *sarA*::*km* (Km^r)	This study
NA3	Clinical strain KS33 *sarA*::*km* (Km^r)	This study
KA1	Clinical strain KS26 with plasmid pKT601 (Tc^r)	This study
KS30.1	8325-4 with *ssp* promoter element from KS30, fused to *lacZ*, inserted in *geh* (Em^r)	This study
KS30.2	8325-4 with mutated *ssp* promoter element from KS30, fused to *lacZ*, inserted in *geh* (Em^r)	This study
Plasmids		
pKT601	*S. aureus* plasmid containing the *sarA* gene under the control of the *xylA* promoter (Tc^r)	50
pKT1	pRZ5202 containing *ermB* and *attP* (Amp^r)	50
pKS30.1	pKT1 with *ssp* promoter::*lacZ* from KS30 (Amp^r)	This study
pKS30.2	PKT1 with mutated (G→A) *ssp* promoter::*lacZ* from KS30 (Amp^r)	This study

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

infusion (BHI) in a 1-liter baffled flask to give an initial optical density at 600 nm (OD_{600}) of 1.0 and were incubated on a rotary shaker (180 rpm) at 37°C.

Preliminary sequence data were obtained from the website of The Institute for Genomic Research (http://www.tigr.org) for *S. aureus* strain COL and from the University of Oklahoma genome sequencing project website (http://www.genome.ou.edu/staph.html) for *S. aureus* strain 8325-4.

PCR. The presence of protease genes in *S. aureus* strains was analyzed by PCR by use of the primers listed in Table 2. Chromosomal DNA from *S. aureus* strains was prepared with the DNeasy Tissue Kit (Qiagen) and was used as a template. PCR fragments were purified with the QiaQuick PCR purification kit (Qiagen) and analyzed on 1% agar gels (Sigma) together with a 1-kb Plus size marker (Life Technologies).

Northern blot analysis. Total *S. aureus* RNA was prepared by using the FAST RNA-blue kit (Bio 101) according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm. Samples containing 10 μg of total RNA were analyzed by Northern blotting as described previously (37). Internal fragments of the 16S rRNA gene (nucleotides [nt] 11 to 1023; GenBank accession no. X68417), the gene coding for RNAIII (nt 1095 to 1578) (28), *sarA* (nt 843 to 1260; GenBank accession no. U46541), *lacZ* (nt 8100 to 8700; GenBank accession no. AE000141), *asp23* (nt 27 to 349; GenBank accession no. S76213), *ssp* (889 bp), *aur* (1,197 bp), and *scp* (407 bp) were amplified by PCR, radiolabeled with [α-^{32}P]dCTP (Amersham) by using a random prime labeling kit (Roche Molecular Biochemicals), and used as probes.

TABLE 2. Primers used for detection of *S. aureus* protease genes by PCR

Primer	Sequence
sspA forward	5′-GAC AAC AGC GAC ACT TGT GA-3′
sspA reverse	5′-AGT ATC TTT ACC TAC AAC TAC A-3′
sspB forward	5′-TGA AGA AGA TGG CAA AGT TAG-3′
sspB reverse	5′-TTG AGA TAC ACT TTG TGC AAG-3′
aur forward	5′-TAG TAG CAC ACG AAT TAA CAC ACG-3′
aur reverse	5′-TTC CCT ATT GCT TGA ATC ACG-3′
scp forward	5′-AAA TTA TTG CAT GCA CTG ATA ATG TGT AA-3′
scp reverse	5′-ATT ACC TTC AGA ATT CAA AAC TGG-3′

Radioactivity was detected by a radioisotope imaging system (PhosphorImager 445SI; Molecular Dynamics).

Analysis of extracellular protein. Total exoproteins from 50 μl of culture supernatant were precipitated with methanol-chloroform (4), dissolved in 10 μl of loading buffer, separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE), and stained with Coomassie brilliant blue according to standard protocols (4). For Western blot analysis of serine protease, culture supernatants corresponding to a bacterial density of 0.12 OD_{600} unit were separated by SDS–12% PAGE and transferred to polyvinylidene difluoride-based membranes (Immobilon-P; Millipore) by using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell as recommended by the supplier. Polyclonal rabbit anti-serine protease antibodies (25) were used and were detected with horseradish peroxidase-conjugated sheep anti-rabbit antibodies (Amersham Life Science).

Inactivation and overexpression of *sarA* in clinical strains. *sarA* was inactivated in clinical *S. aureus* strains KS7, KS30, and KS33 by transfer of the *sarA*::*km* mutation from *S. aureus* strain PC1839 by phage transduction using ϕ11 (39). Transductants (NA1, NA2, and NA3, respectively) were selected on kanamycin (25 μg ml^{-1}) agar plates. Mutations were confirmed by PCR analysis using primers internal to the *sarA* gene and flanking primers in the kanamycin cassette.

Plasmid pKT601, carrying the *sarA* gene under the control of the inducible *xylA* promoter, was transferred to *S. aureus* strain KS26 by electroporation (48). PCR analysis and restriction mapping of the plasmid confirmed the presence of pKT601 in strain KA1. For induction of *sarA*, bacteria were grown on casein agar plates with 0.10% xylose, or in glucose-free BHI containing 0.05% xylose, in the presence of tetracycline (5 μg ml^{-1}).

Construction of chromosomally encoded *ssp* promoter::*lacZ* fusions. The transcription reporter gene vector pKT1, containing the phage L54a attachment site *attPI* (50), was used to generate different *ssp* promoter::*lacZ* fusions. PCR fragments encompassing the promoter region of *ssp* (positions −312 to +14 relative to the transcription start point [12]) were amplified from KS30 chromosomal DNA by using a forward primer (5′-TAA TTG ACT AGT AAA CTT AAG CAC TCA AAT AAT ATA TC-3′) with an added *Spe*I restriction site and a reverse primer (5′-AAA AAT GGA TCC ACA AGT TAA ATA TAA CAA TAA AAA TTT TTA-3′) with an added *Bam*HI restriction site. For construction of pKS30.2, in which the G in position 4 of the −35 box (TTG**G**CT) was replaced by an A (TTG**A**CT), the reverse primer 5′-AAA AAT GGA TCC ACA AGT TAA ATA TAA CAA TAA AAA TTT TTA AGT CAA-3′ was used together with the forward primer described above. The PCR fragments were ligated to pKT1 cut with *Spe*I and *Bam*HI and were used to transform *Escherichia coli* cells. Plasmid constructs were confirmed by nucleotide sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing kit, version 2 (Perkin-Elmer Ap-

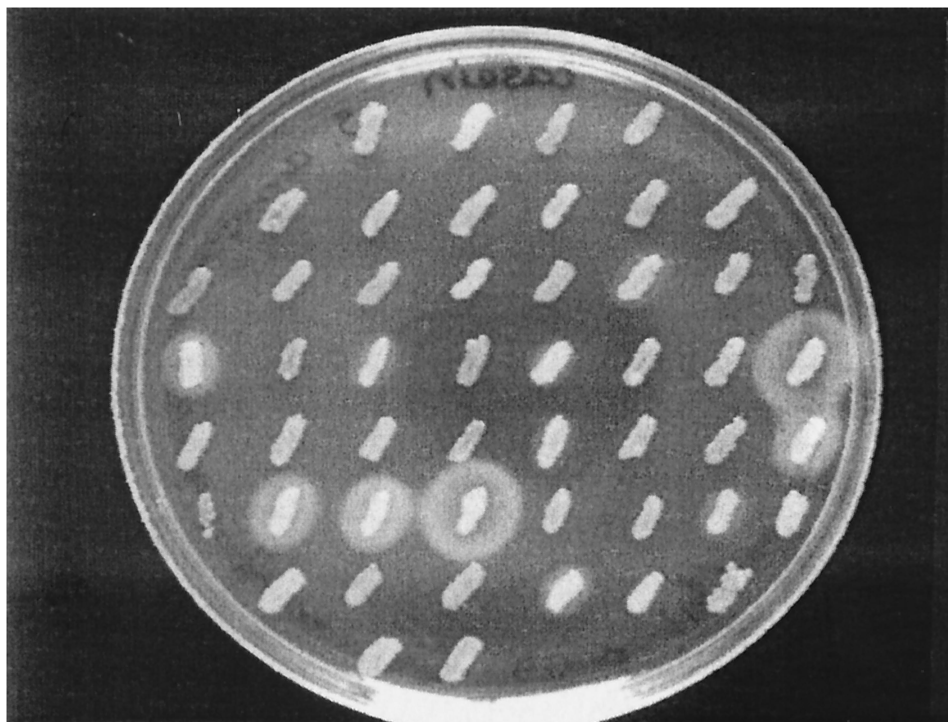

FIG. 1. Zones of proteolysis around 50 clinical isolates of *S. aureus* grown on a casein agar plate.

plied Biosystems), and the Applied Biosystems 377 DNA sequencer. The correct plasmids were then electroporated into *S. aureus* CYL316. Strain CYL316 contains the integrase gene of phage L54a, which allows the plasmid to integrate into the *attB* site of the lipase gene (*geh*). Integrations were confirmed by PCR and were then transduced into *S. aureus* strain 8325-4 by using the transducing phage ϕ11 as described elsewhere (39) to generate strains KS30.1 and KS30.2, respectively. Proper integration of the promoter fusion constructs was confirmed by PCR analysis using a forward primer specific for *geh* and a reverse primer specific for the pKT1 vector.

RESULTS

Expression of extracellular proteases among *S. aureus* clinical isolates. Ninety-two *S. aureus* strains from human soft tissue infections were cultivated on casein agar plates in order to study extracellular protease production. Twenty-one strains (23%) produced a zone of precipitation around the bacterial streak (Fig. 1). Since the quality of the precipitation was most typical for the V8 protease (1), all strains were tested for the presence of the V8 protease gene (*sspA*) by PCR. A PCR product of the expected size (292 nt) was obtained with all strains (data not shown). Twelve protease-negative and five protease-positive strains were also tested for the presence of the other major protease genes. All strains tested were positive for *sspB* (cysteine protease), *aur* (aureolysin), and *scp* (staphopain) (data not shown).

Analysis of expression of the regulators *sarA* and *agr* in protease-negative and protease-positive *S. aureus* strains. A possible explanation for the variation in protease production between strains could be that they express different levels of the protease gene regulators *sarA* (a repressor) and *agr* (RNAIII; an activator). To test this we analyzed the levels of expression of *sarA*, RNAIII, and *ssp* in two protease-positive strains (KS26, a high producer, and KS36, an intermediate producer) and three protease-negative strains (KS7, KS30, and KS33). The level of *ssp* mRNA in each strain correlated roughly with the zone of proteolysis on casein agar plates; it was highest in strain KS26, intermediate in strain KS36, and lowest in the protease-negative strains (Fig. 2). It should be noted that strains KS7 and KS33 produced significant amounts of *ssp* mRNA although they were protease negative on casein agar plates. Western blot analysis of culture supernatants confirmed that KS7 and KS33, as well as KS26, produced serine protease (SspA) (Fig. 3). SspA could not be detected in culture supernatants from strain KS30, which was consistent with the lack of *ssp* mRNA. Serine protease from strains KS7 and KS33 appeared as two bands of higher molecular mass than the corresponding bands from KS26. Most likely, the larger forms of SspA produced by the protease-negative strains represented inactive proforms of the enzyme. The incomplete processing of SspA was consistent with the lack of *aur* expression in KS7 and KS33 (see Fig. 6). Inactivation of *aur* in *S. aureus* strain 8325-4 resulted in complete absence of a zone of proteolysis (27) and in appearance of the larger proforms (data not shown).

An inverse correlation between the levels of *ssp* mRNA and *sarA* mRNA was observed, while there was no obvious correlation between levels of protease production and RNAIII (Fig. 2), suggesting that the variation in protease production between strains might be due to different levels of *sarA* activity. *sarA* is transcribed from three promoters, P1, P2, and P3 (5). P1 and P2 are ordinary sigma factor A (SigA)-dependent promoters, which are expressed mainly during the early exponential phase of growth, while P3 is sigma factor B (SigB) dependent and is expressed during the postexponential and

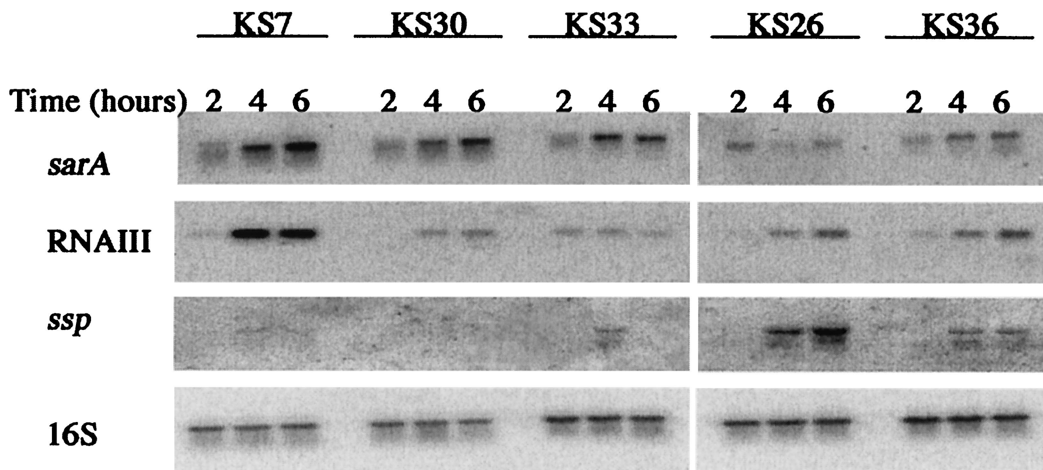

FIG. 2. Northern blot analysis of *sarA*, RNAIII, and *ssp* transcripts in protease-negative (KS7, KS30, and KS33) and protease-positive (KS26 and KS36) clinical isolates of *S. aureus* at different time points during growth. 16S rRNA was used as an internal control of the amount of total RNA loaded. The same filter was hybridized with each of the specific probes.

stationary phases of growth (5, 17, 34). Interestingly, the major *sarA* mRNA in strains KS7, KS30, KS33, and KS36 was the P3 transcript, while the P1 transcript dominated in KS26 (Fig. 2), suggesting reduced *sigB* activity in the latter strain. This was also supported by the observation that transcription of the alkaline shock protein gene *asp23*, which is activated by SigB (20, 22, 30, 36), was severely suppressed in KS26 compared to KS7 (data not shown). In addition, pigmentation, which is also *sigB* dependent (30), was reduced in KS26 compared to that in the protease-negative strains (data not shown). Since *sigB* and *sarA* have been reported to regulate the synthesis of several other secreted proteins in addition to the proteases (19, 21, 51), we analyzed the extracellular-protein patterns of protease-positive and protease-negative *S. aureus* strains by SDS-PAGE. Very different protein patterns were produced (Fig. 4), supporting the hypothesis that these *S. aureus* strains expressed different levels of the global exoprotein regulators *sigB* and *sarA*.

Inactivation and overexpression of *sarA* in clinical strains. To test the hypothesis that the variation in protease production between clinical strains was due to different levels of *sarA* activity, the *sarA* knockout mutation from strain PC1839 was transferred to the protease-negative strains KS7, KS30, and KS33. The resulting *sarA* mutants (NA1 through NA3) produced large zones of proteolysis on casein agar, which were similar in size to that of the clinical isolate with the highest protease production (Fig. 5). The zone of precipitation produced by strain NA2 had a less dense appearance, which is consistent with the lack of V8 protease (see below). Except for the *sspAB* mRNA in strain NA2, levels of protease-specific mRNAs were significantly higher in the *sarA* mutants than in their corresponding wild-type strains (Fig. 6). Notably, transcription of the protease genes increased in the *sarA* mutants, in spite of decreased RNAIII levels. These results indicate that all major protease genes, except for *sspAB* in strain NA2 (see below), were functionally intact in the protease-negative clinical strains and that protease production was repressed due to high levels of *sarA* expression. The complete lack of *aur* mRNA in KS7, KS30, and KS33 indicates that the aureolysin gene is the most sensitive to repression by *sarA*. As seen in Fig. 6, inactivation of *sarA* did not result in constitutive expression of the protease genes. Maximum transcription of *ssp* and *aur* occurred during the postexponential phase of growth (4 h), while expression of *scp* peaked during the exponential phase of

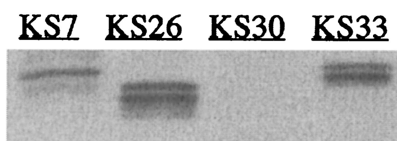

FIG. 3. Western blot analysis of serine protease (SspA) in culture supernatants of *S. aureus* clinical strains. Samples were taken after 4 h of growth.

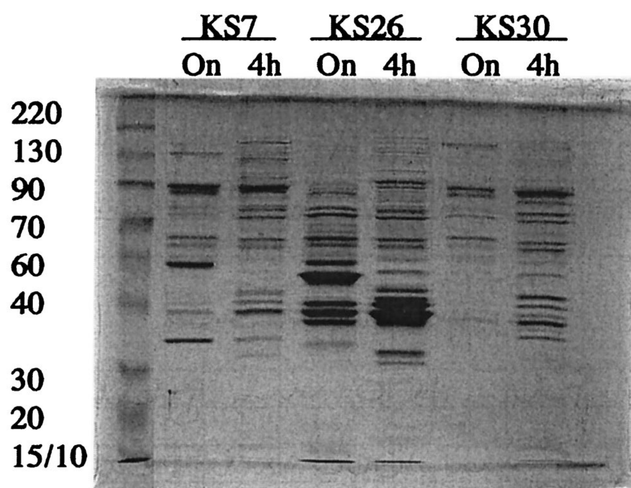

FIG. 4. SDS-PAGE analysis of total extracellular proteins from equal numbers of *S. aureus* cells harvested at the indicated time points. Molecular masses are indicated on the left.

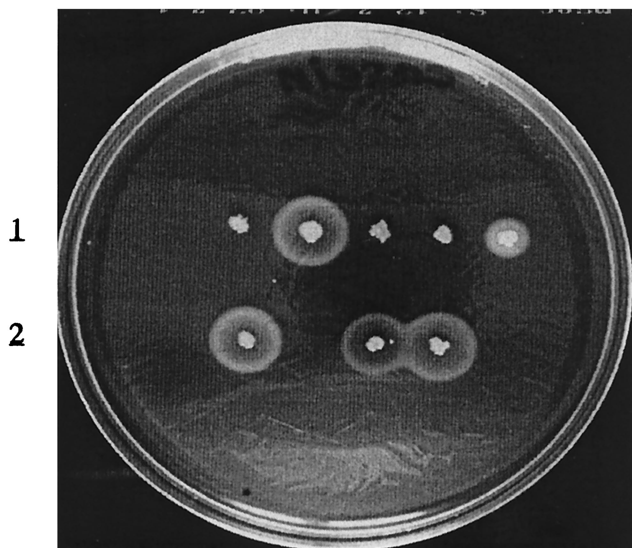

FIG. 5. Protease production by the clinical *S. aureus* strains KS7, KS30, and KS33 and their corresponding *sarA* mutants (NA1, NA2, and NA3) grown on a casein agar plate. The protease-positive strains KS26 and KS36 were included as controls.

growth, indicating that the protease genes are independently regulated in a growth phase-dependent manner. To test the hypothesis that the high level of protease production in strain KS26 was due to a low level of *sarA*, we introduced *sarA* under the control of the inducible *xylA* promoter in strain KS26. Induction of *sarA* transcription with xylose (Fig. 7B) completely repressed protease production (Fig. 7A). Since the level of RNAIII was unaffected (data not shown), the decrease in protease production was most likely a direct effect of increased *sarA* transcription.

Analysis of the *ssp* promoter in strain KS30. To explain the lack of *ssp* transcription in the *sarA* mutant derived from strain KS30, we decided to study the *ssp* promoter of this strain in more detail. Nucleotide sequencing of the *ssp* promoter region in strain KS30 and 10 other protease-negative strains revealed a G at position 4 of the −35 promoter element (TTG**G**CT) in strain KS30 as opposed to an A (TTG**A**CT) in all other strains, including four published sequences (The Institute for Genomic Research and University of Oklahoma websites [see Materials and Methods]) from protease-positive strains. This suggests that the point mutation in the −35 box of strain KS30 might be responsible for the lack of *ssp* transcription. However, minor differences in the nucleotide sequences upstream of the −35 box could also be responsible for the lack of *ssp* expression in strain KS30. To test this, *ssp* promoter DNA fragments (−312 to +14) from strain KS30 with either a G or an A in the −35 box were fused to a promoterless *lacZ* gene. These constructs were integrated as single copies into the lipase gene (*geh*) of the protease-positive prototype *S. aureus* strain 8325-4, and transcription of *lacZ* was analyzed. The corresponding *ssp* promoter fragment of strain 8325-4 was used as a control (50). As shown in Fig. 8, the original *ssp* promoter of KS30 was inactive, while the mutated promoter was almost as active as that of the control. These results suggest that the mutation in the −35 box of the *ssp* promoter in strain KS30 was responsible for the lack of *ssp* transcription and that nucleotide differences upstream of −35 were less important.

DISCUSSION

In the present study we found that production of extracellular proteases varied considerably among clinical isolates of *S. aureus*. The presence of the major protease genes, *sspA*, *sspB*, *aur*, and *scp*, in all the protease-negative strains analyzed suggested that the lack of protease production was due to some regulating host cell factor.

Analysis of the protease regulators *agr* (an activator) and *sarA* (a repressor) in protease-negative and protease-positive strains indicated that protease production in vitro was primarily determined by the level of *sarA* expression. This was

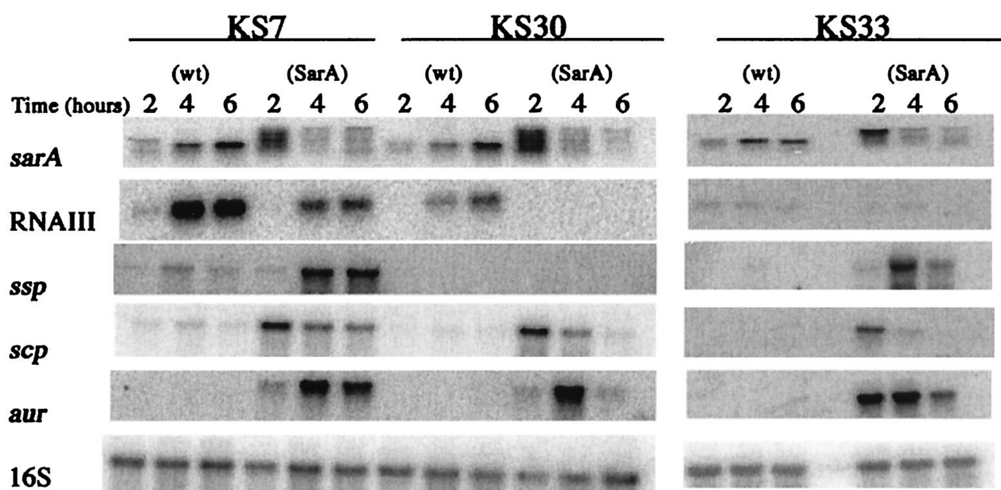

FIG. 6. Northern blot analysis of *sarA*, RNAIII, *ssp*, *scp*, and *aur* in the protease-negative clinical *S. aureus* strains and their corresponding *sarA* mutants. 16S rRNA was used as an internal control of the amount of total RNA loaded. The same filter was hybridized with each of the specific probes. KS33 and its *sarA* mutant were on a separate membrane.

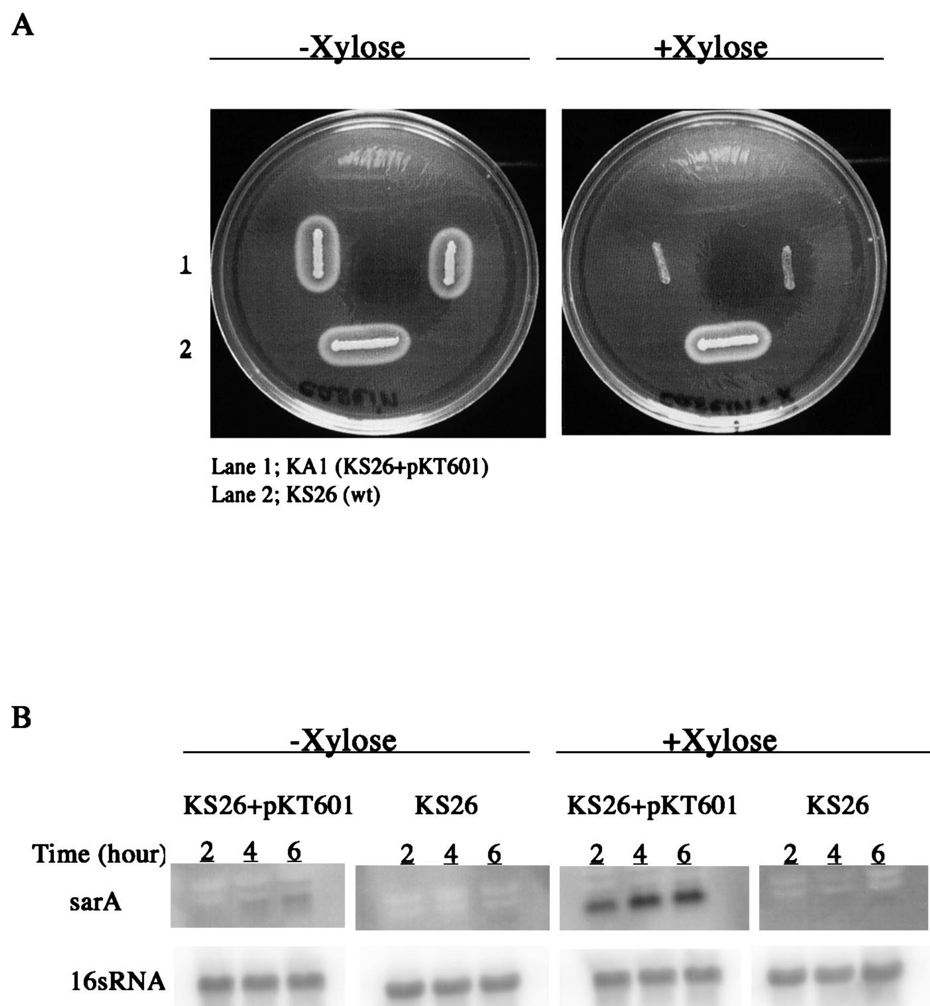

FIG. 7. (A) Effect of induction of *sarA* expression in strain KA1, containing plasmid pKT601, on protease production analyzed on a casein agar plate. (B) Northern blot analysis of *sarA* transcription after induction with xylose in liquid culture.

strongly supported by the demonstration that inactivation of *sarA* in three different protease-negative strains resulted in increased transcription of the protease genes, while overexpression of *sarA* in a protease-positive strain completely inhibited protease production. These experiments also show that the protease genes were functionally intact in the clinical *S. aureus* strains and responded normally to repression by *sarA*.

Although expression of all major protease genes was down-regulated in the protease-negative strains, transcription of *aur* seemed to be the most sensitive to repression by *sarA* (Fig. 6). Since aureolysin is required for activation of the staphylococcal serine protease, SspA (18), down-regulation of *aur* would lead to the loss of both aureolysin and serine protease activity. We have previously shown that inactivation of the aureolysin gene in the *sarA* mutant PC1839, which produces large amounts of all major proteases, resulted in a protease-negative phenotype (27). Accordingly, lack of aureolysin expression would explain the protease-negative phenotype of strain KS7 in spite of the fact that it has relatively high levels of *ssp* mRNA. The identification of nonprocessed serine protease in culture supernatants of KS7 supported this explanation.

The high *sarA* expression in protease-negative strains was due to significant transcription of *sarA* from its SigB-dependent promoter, P3, suggesting that protease-negative strains had higher SigB activity than protease-positive strains. This was supported by the observation that other SigB-dependent traits, such as expression of *asp23* and pigmentation (20, 22, 30, 36), were also higher in protease-negative strains. Since protease production was repressed in the majority of clinical *S.*

FIG. 8. Northern blot analysis of *lacZ* in *S. aureus* 8325-4 containing different *ssp* promoter elements (nt −312 to +14) fused to *lacZ* as a single copy in the chromosomal lipase gene, *geh*. Samples were taken after 4 h of growth, and equal amounts of RNA were analyzed for each sample. Strain KT111 has the *ssp* promoter element from strain 8325-4 (−35 box, TTGACT), KS30.1 has the *ssp* promoter element from strain KS30 (−35 box, TTGGCT), and KS30.2 has the *ssp* promoter element from strain KS30 with a G-to-A substitution at position 4 in the −35 box (TTGACT).

aureus strains one might assume that high SigB activity, and therefore high *sarA* activity, is the normal phenotype under in vitro growth conditions. Strains with high protease production seemed to have low SigB activity. In the protease-positive *S. aureus* strain 8325, SigB-dependent expression of *sarA* is decreased because of a deletion in the SigB activator gene *rsbU* (22, 30, 41). Whether the protease-positive clinical isolates have a similar SigB defect remains to be determined.

Interestingly, there was no correlation between protease production and the level of the activator RNAIII (*agr*) in the clinical strains, indicating that the negative effect of *sarA* on protease production is dominant over the stimulating effect of RNAIII. This was also supported by the observation that transcription of protease genes increased in the *sarA* mutants, in spite of reduced RNAIII levels (Fig. 6). The observed reduction of RNAIII levels in *sarA* mutants is consistent with previous reports suggesting that *sarA* is an activator of *agr* transcription (10, 15, 19, 23). However, on the other hand, overexpression of *sarA* in the protease-positive clinical strain KS26 had no significant effect on RNAIII production (data not shown), suggesting that other host cell factors modulate the regulation of RNAIII expression. Bischoff et al. (6) found that induction of *sigB* increased the expression of *sarA* but decreased the level of RNAIII. They suggested that SigB induced a repressor with a dominating effect over the *sarA*-dependent activation of *agr* transcription. An alternative explanation would be that SarA at very high concentrations acts as a repressor of *agr*. The function of SarA as a repressor has been demonstrated in an in vitro transcription system with *S. aureus* RNA polymerase (13).

All protease genes in the clinical *S. aureus* strains investigated seemed to be intact and reacted normally to SarA except for *ssp* in KS30, which was not up-regulated in response to inactivation of *sarA*. This was shown to be due to an A-to-G substitution at position 4 in the −35 hexanucleotide box (TTGACT) of the *ssp* promoter. The same base substitution in the *Salmonella ant* promoter resulted in a 30-fold reduction in promoter activity (38). As the *ssp* operon was silent in KS30, the zone of proteolysis produced by the *sarA* mutant NA2 is most likely due to staphopain and/or aureolysin. Together with the observation that an aureolysin mutant was protease negative on casein agar (27), this suggests that staphopain is activated by aureolysin.

Our results show that expression of the major protease genes is down-regulated in most clinical *S. aureus* strains under in vitro cultivation conditions due to high SigB-dependent *sarA* activity. Considering the potential role of the extracellular proteases in staphylococcal virulence, it must be assumed that they are produced at some point during infection. This means that SigB activity must be down-regulated under in vivo growth conditions or that an activator that has a dominating effect over the SarA-dependent repression of protease production must be produced. The same type of regulation would be needed for the in vivo expression of other virulence factors that are repressed by *sarA* and/or SigB, e.g., lipase, staphylokinase, alpha- and beta-hemolysins, leukotoxin, and collagen-binding protein D (9, 51). On the other hand, a number of virulence factors are activated by SigB and/or *sarA* (19, 51), which means that the bacteria must still be able to increase the levels of *sigB* and *sarA* expression during infection. Therefore, the ability to regulate the expression of virulence factors in a proper way is probably much more important than the virulence factor profile expressed in vitro. However, it remains to be proven whether protease-negative and protease-positive clinical *S. aureus* strains are equally virulent, although they were isolated from apparently similar type of infections.

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