4-2005

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JOURNAL OF BACTERIOLOGY, Apr. 2005, p. 2348–2356 Vol. 187, No. 7
0021-9193/05/$08.00+0 doi:10.1128/JB.187.7.2348–2356.2005

SarA Is an Essential Positive Regulator of *Staphylococcus epidermidis* Biofilm Development

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Received 2 September 2004/Accepted 23 December 2004

Staphylococcus epidermidis **biofilm formation is associated with the production of the polysaccharide intercellular adhesin (PIA)--poly-*N*-acetylglucosamine polysaccharide (PNAG) by the products of the *icaADBC* operon. Recent evidence indicates that SarA, a central regulatory element that controls the production of *Staphylococcus aureus* virulence factors, is essential for the synthesis of PIA/PNAG and the ensuing biofilm development in this species. Based on the presence of a *sarA* homolog, we hypothesized that SarA could also be involved in the regulation of the biofilm formation process in *S. epidermidis*. To investigate this, we constructed nonpolar *sarA* deletions in two genetically unrelated *S. epidermidis* clinical strains, O-47 and CH845. The SarA mutants were completely defective in biofilm formation, both in the steady-state conditions of a microtiter dish assay and in the flow conditions of microfermentors. Reverse transcription-PCR experiments showed that the mutation in the *sarA* gene resulted in downregulation of the *icaADBC* operon transcription in an IcaR-independent manner. Purified SarA protein showed high-affinity binding to the *icaA* promoter region by electrophoretic mobility shift assays. Consequently, mutation in *sarA* provoked a significant decrease in the amount of PIA/PNAG on the cell surface. Furthermore, heterologous complementation of *S. aureus sarA* mutants with the *sarA* gene of *S. epidermidis* completely restored biofilm formation. In summary, SarA appeared to be a positive regulator of transcription of the *ica* locus, and in its absence, PIA/PNAG production and biofilm formation were diminished. Additionally, we present experimental evidence showing that SarA may be an important regulatory element that controls *S. epidermidis* virulence factors other than biofilm formation.**

Chronic nosocomial infections by biofilm-forming *Staphylococcus epidermidis* have become more prevalent in recent years with the increased use of prosthetic medical implants. Biofilm formation by *S. epidermidis* frequently compromises the effectiveness of implanted medical devices by giving rise to persistent and relapsing infections, which are more resistant to the host immune response and antimicrobial chemotherapy (for a review, see reference 18). The formation of *S. epidermidis* biofilms is proposed to occur in a two-step manner, in which a cellular accumulation process to form the mature biofilm follows rapid initial attachment to an inert synthetic surface (22). Critical to *S. epidermidis* biofilm formation is the production of a poly-*N*-acetylglucosamine polysaccharide (PNAG)-polysaccharide intercellular adhesin (PIA) (33, 34). The intercellular adhesin (*icaADBC*) locus, originally described in *S. epidermidis* (22, 23) and later found in *Staphylococcus aureus* (9), contains the genes involved in PIA/PNAG production. The significance of PIA/PNAG as a virulence factor was demonstrated in a central venous catheter infection model of a rat and in a subcutaneous foreign-body infection model in mice (43, 44). In addition, the *ica* operon is one of the few genetic markers of *S. epidermidis* that differs between invasive strains and strains from the skin flora (15, 16).

PIA/PNAG production and biofilm formation in *S. epidermidis* are regulated by a variety of environmental factors, including high osmolarity (3% NaCl), ethanol (4%), glucose, growth in anaerobic conditions, high temperature, and subinhibitory concentrations of certain antibiotics (7, 10, 12, 27, 28). Genetic regulation involving *icaR* and the alternative sigma factor σ^B has been reported. The gene *icaR*, located adjacent to the *ica* operon, encodes a transcriptional repressor involved in the environmental regulation of *icaADBC* operon expression in *S. epidermidis* and *S. aureus* (8, 24, 25). A transcriptional analysis revealed that *icaADBC* transcription was strongly repressed in mutants with defective σ^B, whereas *icaR* was upregulated, a finding suggesting that σ^B controls transcription of the *icaADBC* operon by an *icaR*-dependent pathway in *S. epidermidis* (27, 28). However, the alternative transcription factor σ^B plays contradictory roles in controlling biofilm formation of *S. aureus*. While an initial work by Rachid and coworkers implied that σ^B was a regulator of the biofilm formation process of *S. aureus* (42), our results have demonstrated that biofilm and PIA/PNAG production were not affected in a *S. aureus* σ^B mutant compared with its wild-type strain (47). Moreover, *ica* operon expression in *S. epidermidis* can be turned on and off by the insertion and excision of the insertion sequence IS*256* at specific hot spots in the *icaA* and *icaC* genes

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Species	Relevant properties	Source or reference
Strains			
RN4220	*S. aureus*	Restriction-deficient mutant of 8325-4	29
15981	*S. aureus*	Clinical isolate, biofilm-positive	47
15981 Δ*ica*	*S. aureus*	Derivative of 15981; *icaADBC*-negative, biofilm-negative strain	47
15981 Δ*sarA*	*S. aureus*	Derivative of 15981; *sarA*-negative, biofilm-negative strain	47
ISP479c	*S. aureus*	Derivative of 8325; biofilm-positive strain	40
ISP479c *sarA*-	*S. aureus*	Derivative of ISP479c; *sarA*-negative; biofilm-negative strain	47
O-47	*S. epidermidis*	Clinical strain; *icaADBC*-positive; biofilm-positive strain	22
O-47 Δ*ica*::*tet*	*S. epidermidis*	Derivative of O-47; *icaADBC* mutant; biofilm-negative strain	10
CH845	*S. epidermidis*	Clinical strain BM94314; *icaADBC*-positive; biofilm-positive strain	16
JP54	*S. aureus*	15981 Δ*sarA*(pJP19)	This study
JP55	*S. aureus*	ISP479c *sarA*(pJP19)	This study
JP56	*S. epidermidis*	Derivative of O-47; *sarA* mutant	This study
JP57	*S. epidermidis*	Derivative of CH845; *sarA* mutant	This study
JP58	*S. epidermidis*	JP56(pJP19)	This study
JP59	*S. epidermidis*	JP57(pJP19)	This study
Plasmids			
pCU1		Shuttle plasmid	2
pMAD		Shuttle vector with a termosensitive origin of replication for gram-positive bacteria	1
pJP18		Derivative of pMAD used to construct the deletions in the *sarA* gene	This study
pJP19		Vector for complementation experiments; a 1.1-kb PCR fragment containing *sarA* from *S. epidermidis* RP62A cloned in pCU1	This study

(51). By this mechanism, PIA/PNAG production and biofilm formation phenotypes may be phase variable.

By screening a library of Tn*917* insertions in a clinical *S. aureus* strain, we identified SarA as being essential for biofilm development by *S. aureus* (47). Nonpolar mutations of *sarA* in genetically unrelated *S. aureus* strains decreased *ica* transcription and PIA/PNAG production and completely prevented biofilm development. In *S. epidermidis*, a SarA protein has been described (14). This protein is highly related (84%) to the *S. aureus* SarA protein, suggesting that it could be involved in the control of virulence determinants. However, no data on *sar* regulation exist, and some evidence suggests the existence of particularities for each species. Thus, the order of the three promoters that control *sarA* expression is not the same in *S. epidermidis* as in *S. aureus*, a distinction which might reflect differences in regulation (14).

In this study, we have examined the role of SarA in the regulation of *icaADBC* operon expression, PIA/PNAG production, and biofilm formation in two genetically unrelated biofilm-positive clinical isolates of *S. epidermidis*. Our results demonstrate that the *sarA* mutants show a severe defect in biofilm formation through a transcriptional downregulation of *icaADBC* operon expression and PIA/PNAG production by an IcaR-independent pathway.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The biofilm-forming strain *S. epidermidis* O-47 was isolated from a patient at the Institut für Medizinische Mikrobiologie und Hygiene, Universität zu Köln, Cologne, Germany (22). *S. epidermidis* CH845 was isolated from a patient with infection in a joint prosthesis (16). Both strains were selected because of their strong biofilm production phenotype, antibiotic susceptibility profile, and ability to accept recombinant DNA by protoplast transformation.

The most relevant bacterial strains and plasmids used and constructed in this study are listed in Table 1. *Escherichia coli* DH5α cells were grown in Luria-Bertani (LB) broth or on LB agar (Pronadisa) with appropriate antibiotics. Staphylococcal strains were cultured on Trypticase soy agar (TSA), in Trypticase soy broth supplemented with glucose (0.25% wt/vol; TSB-gluc), and in Congo red agar (11). Media were supplemented with appropriate antibiotics in the following concentrations: erythromycin, 2.5 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 20 μg/ml.

DNA manipulations. Routine DNA manipulations were performed with standard procedures (45) unless otherwise stated. Plasmid DNA from *E. coli* and staphylococci were purified with a Genelute plasmid miniprep kit (Sigma) according to the manufacturer's protocol, except that the staphylococcal bacterial cells were lysed by lysostaphin (Sigma; 12.5 μg/ml) at 37°C for 1 h before plasmid purification. Plasmids were introduced into the staphylococci by electroporation or by protoplast transformation with previously described methods (11, 19, 20). Restriction enzymes were purchased from Roche and used according to the manufacturer's instructions. Oligonucleotides were obtained from Invitrogen (Table 2).

Staphylococcal chromosomal DNA was extracted with a Genelute bacterial genomic DNA kit (Sigma) according to the manufacturer's protocol, except that the bacterial cells were lysed by lysostaphin (Sigma; 12.5 μg/ml) at 37°C for 1 h before DNA purification. For Southern blot hybridization, the chromosomal DNA digested with HindIII was analyzed by agarose gel electrophoresis. Gels were blotted onto nylon membranes (Hybond-N 0.45-mm-pore-size filters, Amersham Life Science) with standard methods (3, 45). The PCR product of the amplified *sarA* gene with oligonucleotides Sarepi-5c and Sarepi-8c was used as a DNA probe. Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labeling and chemiluminescent detection kit (Roche).

Allelic exchange of chromosomal genes. To construct the deletions in *sarA*, we amplified by PCR two fragments of approximately 1,000 bp that flanked the left (oligonucleotides Sarepi-4cB and Sarepi-3m) and the right (oligonucleotides Sarepi-2c and Sarepi-1mX) of the sequence targeted for deletion (Table 2). Oligonucleotides Sarepi-2c and Sarepi-3m have a 20-base complementary region (underlined in the oligonucleotide sequence) to allow the products of the first PCR to anneal at their overlapping region. A second PCR was performed with primers Sarepi-1mX and Sarepi-4cB to obtain a single fragment. Specifically, 1 μl of each of the products of the first PCR was mixed with 10 pM of the outside primers and PCR amplified. The fusion products were purified and cloned into the BamHI and XbaI sites of plasmid pSC20 (F. Götz). The fragment was then cloned into the SalI and EcoRI sites of the shuttle plasmid pMAD (1), and the resulting plasmid, pJP18, was transformed into *S. epidermidis* by protoplast transformation. Plasmid pMAD contains a temperature-sensitive origin of rep-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′-3′)
Sarepi-1mX	TGCTCTAGAATGACTAAAGGGAGGTGCC
Sarepi-2c	GGCTAGGGAGTAAAACAGATATTTC
Sarepi-3m	ATCTGTTTTACTCCCTAGCCGCAATGTAGC ATTTGCTATATTC
Sarepi-4cB	CGCGGATCCTAAATTAACTTCTAAAACA GAAG
Sarepi-5c	TGGATATGATATAAATAGGGAGG
Sarepi-6cX	TGCTCTAGAGGACATGCACCACATATC GAGG
Sarepi-7mB	CGCGGATCCGGTATATTAATATAACTAA AGGC
Sarepi-8c	TCTGTGATACGGTTGTTTACTCG
icaA-epi-1	AACAAGTTGAAGGCATCTCC
icaA-epi-2	GATGCTTGTTTGATTCCCT
icaR-epi-1	GGTAAAGTCCGTCAATGGAA
icaR-epi-2	CGCAATAACCTTATTTTCCG
icaR-aur-1c	ATTGCGTTATCAATAATCTTATC
icaA-aur-3c	TTGCAATTTCTTTACCTACCTTTCG
icaR-epi-7c	ATTGCGTTATCAATAATCTTATC
icaA-epi-3	CATGCATTTTTTCACCTACCTTTCG
gyrB-epi-1	TTATGGTGCTGGACAGATACA
gyrB-epi-2	CACCGTGAAGACCGCCAGATA

lication and an erythromycin resistance gene. The plasmid was integrated into the chromosome through homologous recombination at the nonpermissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into 10 ml of TSB-gluc and incubated for 24 h at 30°C. Tenfold serial dilutions of this culture in sterile TSB-gluc were plated on TSA containing 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside at 150 μg ml^{-1}). White colonies, which no longer contained the pMAD plasmid, were tested to confirm the replacement by PCR with oligonucleotides Sarepi-1mX and Sarepi-5c (Table 2) and by Southern blotting.

Complementation of the mutants. The *sarA* gene from *S. epidermidis* RP62A was amplified with high-fidelity thermophilic DNA polymerase (Dynazyme Ext, Finnzymes) with primers Sarepi-7mB and Sarepi-6cX (Table 2). The PCR product was cloned into the BamHI and XbaI sites of the pCU1 plasmid (2), and the resulting plasmid, pJP19, was transformed by electroporation or protoplast transformation into *S. aureus* RN4220 or *S. epidermidis* strains. Phage 80α was used to transduce pJP19 from RN4220 to strains 15981 and ISP479c (39).

Biofilm formation assays. The quantification of the biofilm formation on abiotic surfaces was assessed basically as described elsewhere (11, 22). Macroscopic observation of biofilm on glass was performed as previously described (11). Briefly, cells were grown in 50 ml of B2 at 37°C, with a glass container, without shaking, for 1 day, and the walls of the container were visually (macroscopically) examined for the presence or absence of a white biofilm layer.

To analyze biofilm formation under flow conditions, we used 60-ml microfermenters (Pasteur Institute's Laboratory of Fermentation) with a continuous flow of 40 ml h^{-1} of TSB-gluc and constant aeration with sterile compressed air (0.3 bar). Submerged Pyrex slides served as the growth substratum. Approximately 10^8 bacteria from an overnight preculture of each strain grown in TSB-gluc were used to inoculate the microfermenters and cultivated 24 h at 37°C. Biofilm development was recorded with a Nikon Coolpix 950 digital camera.

PNAG detection. PNAG production in the *S. epidermidis* strains was detected as described elsewhere (9), with an anti-*S. aureus* PNAG antibody diluted 1:10,000 (34). Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Santa Cruz) diluted 1:5,000, and the Western blotting Luminol reagent (Santa Cruz Biotechnology).

Real-time quantitative PCR. Total *S. epidermidis* RNA was prepared with the Fast RNA-Blue kit (Bio 101) according to the manufacturer's instructions. Two micrograms of each RNA was subjected, in duplicate, to DNase I (Invitrogen) treatment for 30 min at 37°C. The enzyme was inactivated at 65°C in the presence of EDTA. To verify the absence of genomic DNA in every sample, the RNA duplicates were reverse transcribed in the presence and absence of Moloney murine leukemia virus reverse transcriptase (Invitrogen). All preparations were purified with the QIAquick PCR purification kit (Qiagen); 25 ng of each reaction product was used for a real-time quantitative PCR with the iCycler machine (Bio-Rad) and the LC-DNA Master SYBR Green I mix (Bio-Rad). The *icaA* and *icaR* transcripts were amplified with primers icaA-epi-1/icaA-epi-2 and icaR-epi-1/icaR-epi-2, respectively (Table 2). The *gyrB* transcripts that are constitutively expressed were amplified as an endogenous control with the primers gyrB-epi-1 and gyrB-epi-2 (Table 2). The level of expression of *icaA* was normalized with respect to *gyrB* expression. Only samples with no amplification of *gyrB* in the minus-reverse transcriptase aliquot were included in the study.

To monitor the specificity, the final PCR products were analyzed by melting curves and electrophoresis. In each experiment, all the reactions were performed in triplicate. The relative transcriptional levels within distinct experiments were determined with the $2^{-\Delta\Delta CT}$ method (31). The results show the average ± standard error of the mean of at least four independent experiments.

Purification of SarA protein. The cloning and purification of the His_6-tagged SarA fusion protein were described earlier (6). The purified His_6-tagged SarA protein was found to be more than 98% pure in a sodium dodecyl sulfate–12% polyacrylamide gel. The concentration of the purified protein was determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.), with bovine serum albumin as the standard.

Electrophoretic mobility shift. To determine if the recombinant SarA protein from *S. aureus* binds to the *icaA* promoter region, a 200-bp PCR-amplified fragment, representing the *icaRA* intergenic region from *S. aureus* (oligonucleotides icaR-aur-1c and icaA-aur-3c) or *S. epidermidis* (oligonucleotides icaR-epi-7c and icaA-epi-3) was end labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. Labeled fragment (0.1 ng or 0.5 fmol) was incubated at room temperature for 20 min with various amounts of purified SarA protein in 25 μl of binding buffer (25 mM Tris-Cl [pH 7.5], 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 μg of calf thymus DNA (Amersham Pharmacia Biotech). The reaction mixtures were analyzed in an 8.0% nondenaturing polyacrylamide gel. The band shifts were detected by exposing dried gels to X-ray films.

Statistical analysis. The data indicating gene expression were compared with the Kruskal-Wallis and the Mann-Whitney tests. All the tests were two-sided, and the significance level was 5%. The statistical analysis was performed with the SPSS program.

RESULTS

***sarA* gene of *S. epidermidis* restored the biofilm formation capacity of the *S. aureus* Δ*sarA* mutants.** Considering the similarity of the SarA proteins of *S. aureus* and *S. epidermidis*, we investigated the functional relationship of the *sarA* gene in both species. The *S. aureus sarA* mutant clones 15981 Δ*sarA* and ISP479c *sarA* mutant (47) complemented with plasmid pJP19 (carrying a PCR-amplified 1,109-bp fragment containing the *sarA* gene from *S. epidermidis* under the control of its own promoter) were analyzed for their capacity to form a biofilm. As shown in Fig. 1, we found that the complemented strains JP54 and JP55 regained their capacity to form biofilms compared with their respective *sarA* mutants. Evidently, the SarA protein of *S. epidermidis* is functional in *S. aureus*.

Deletion of *sarA* in *S. epidermidis* resulted in a reduced capacity to form a biofilm in vitro. To assess the role of SarA in *S. epidermidis*, we constructed nonpolar deletions of the *sarA* gene in two unrelated *S. epidermidis* clinical strains by allelic exchange with the pMAD plasmid (see Materials and Methods). As shown in Fig. 2A and B, the deletion mutants JP56 (*S. epidermidis* O-47 Δ*sarA*) and JP57 (*S. epidermidis* CH845 Δ*sarA*) were reduced in their capacity to form a biofilm on polystyrene microtiter plates compared to the wild-type parent strains O-47 and CH845, respectively. When the JP56 strain was complemented with plasmid pJP19, carrying the wild-type *sarA* gene, biofilm formation was restored (Fig. 2A and B). In contrast, although strain JP59 (JP57 carrying plasmid pJP19) formed large clumps in broth cultures, it was unable to form a biofilm on microtiter plates (data not shown). However, macroscopic examination of biofilm formation in a glass container revealed that upon 1 day of culture, the wild-type (O-47 and

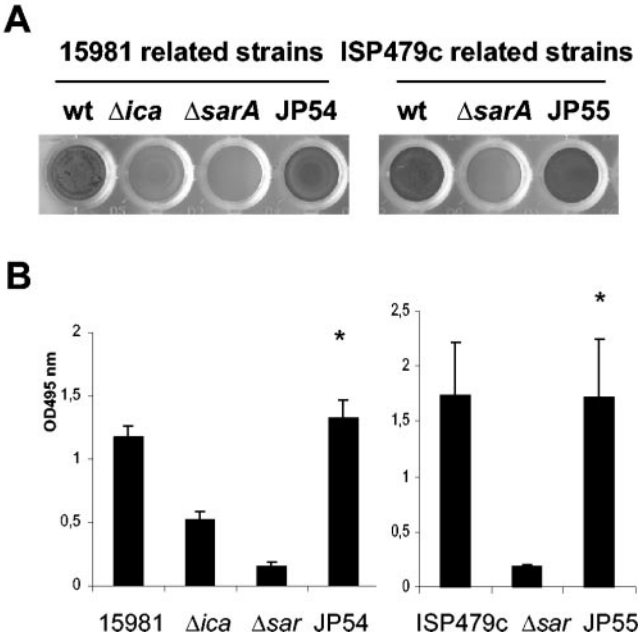


FIG. 1. Biofilm formation phenotype of two unrelated *S. aureus sarA* mutant clones carrying pJP19, a shuttle plasmid containing the *sarA* gene from *S. epidermidis*. Biofilm formation capacity differences correspond to 24-h biofilm formed on polystyrene microtiter plates after staining with 0.1% safranin. The microtiter plates and mean optical density values obtained (A_{495}) are shown. (A) Left: wells corresponding to wild-type 15981, 15981 Δ*ica* (negative control), 15981 Δ*sarA*, and JP54 (15981 Δ*sarA* carrying pJP19). Right: wells corresponding to wild-type ISP479c, ISP479c Δ*sarA*, and JP55 (ISP479c Δ*sarA* carrying pJP19). (B) Mean optical density values. Bars represent the mean values, and error bars represent the standard error of the mean. Significant differences in adherence were noted between complemented and noncomplemented *sarA* mutant strains (*, $P < 0.01$).

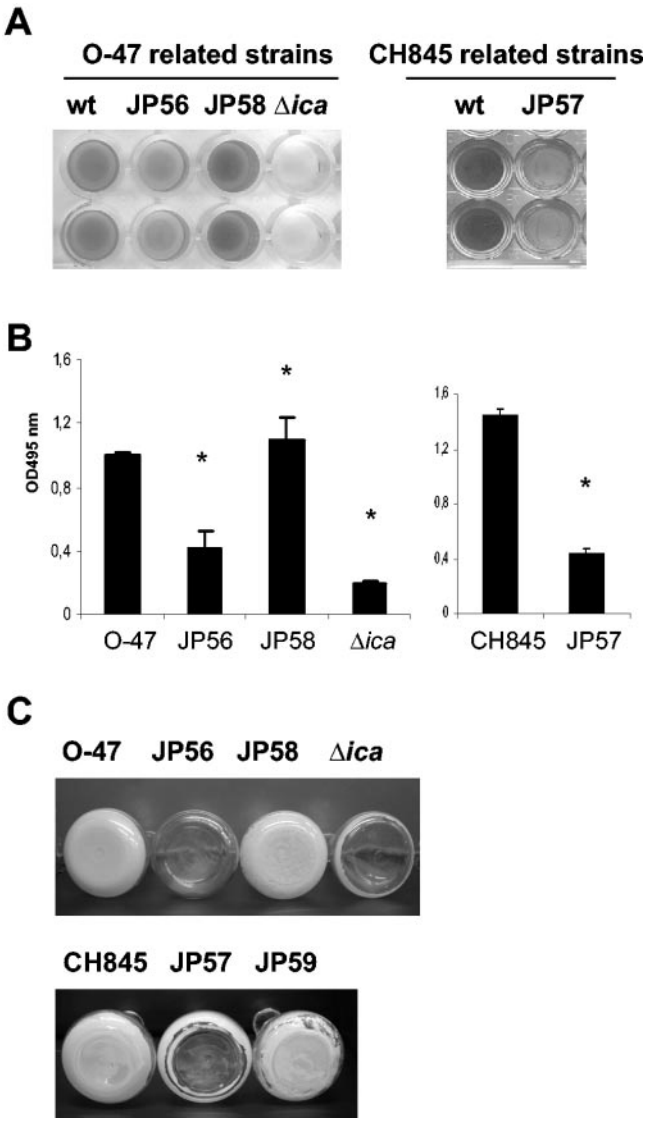


FIG. 2. Loss of biofilm formation in two genetically unrelated *S. epidermidis sarA* mutants. (A) Biofilm formation capacity of *S. epidermidis* O-47 and CH845 (wild-type strains), their corresponding *sarA* mutants JP56 and JP57, the JP56 strain complemented with plasmid pJP19 (JP58), and O-47 Δ*ica*::*tet* as a negative control on polystyrene microtiter plates after 24 h in TSB-gluc medium at 37°C. The bacterial cells were stained with safranin and quantified by determining the absorbance at 495 nm. (B) Significant differences in adherence were noted between wild-type strains and their isogenic *sarA* mutants as well as between the complemented versus noncomplemented JP56 (O-47 *sarA* mutant) strain (*, $P < 0.05$). (C) Phenotypic differences in the capacity to form a 24-h biofilm on the surface of a glass container (visual observation) between wild-type strains O-47 and CH845, their corresponding *sarA* mutants (JP56 and JP57, respectively), and their *sarA* mutants complemented with plasmid pJP19 (JP58 and JP59, respectively).

CH845) and the complemented (JP58 and JP59) strains formed an obvious biofilm on the glass surface, whereas the *sarA* mutants JP56 and JP57 did not (Fig. 2C). These results strongly suggest that the *sarA* gene is required for biofilm formation in *S. epidermidis*.

Biofilm formation of the *sarA* mutants in continuous-flow culture microfermenters. Extracellular proteases, including extracellular cysteine (Ecp) and serine (Esp) proteases, have been described in *S. epidermidis* (13). The first enzyme shows extended sequence similarity to the *S. aureus* cysteine protease (staphopain), and the second resembles the serine protease produced by that species. In *S. aureus*, the expression of the extracellular proteases is repressed by SarA, since their production is upregulated in *sarA* mutants (5). In *S. epidermidis*, nothing is known about their regulation, but it is likely that a similar control by SarA occurs. To test that, the proteolytic activity of the *sarA* mutant JP56 and JP57 strains and their parental strains was analyzed on 1.5% skimmed milk agar plates. As shown in Fig. 3A, the larger proteolytic halo around the *sarA* mutant colonies of JP56 and JP57 compared with that in the wild-type strains indicated an enhanced capacity of these strains to produce extracellular proteases and suggested that SarA is a repressor of protease production in *S. epidermidis*.

Thus, we wondered whether the decreased biofilm formation by the *sarA* mutant strains could be the consequence of the accumulation of extracellular proteases in the microtiter plates and the degradation of a surface protein required for biofilm formation. To examine this possibility, we used microfermenters, where the medium is continuously replenished (17). As shown in Fig. 3B, the wild-type strain O-47 adhered abundantly to the submerged Pyrex spatula and, after 24 h, formed a thick

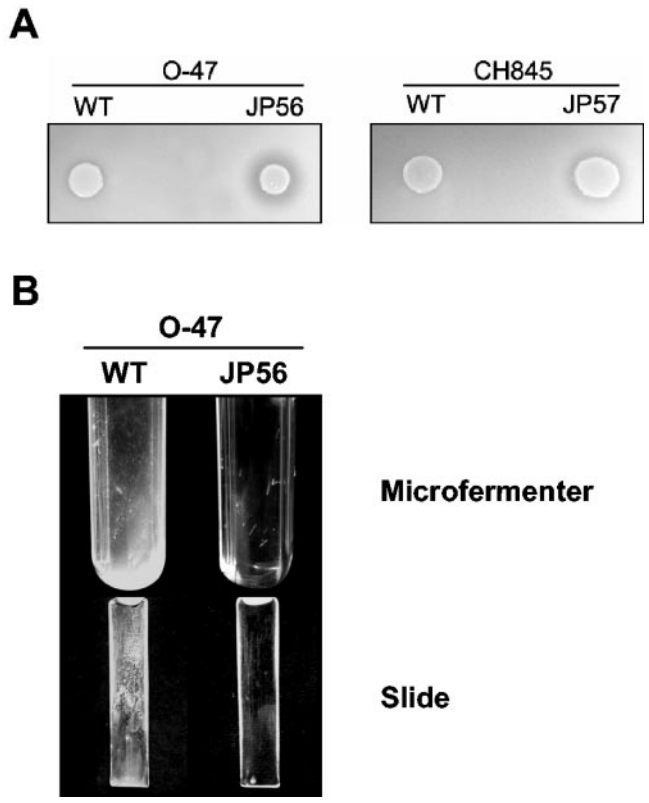


FIG. 3. (A) Increased protease production by *S. epidermidis sarA* mutants. *S. epidermidis* wild-type strains O-47 and CH845 and their corresponding *sarA* mutants were grown in skimmed milk agar plates. (B) Biofilm formation in continuous-flow culture microfermenters of *S. epidermidis* O-47 and its derivative *sarA* mutant JP56. Biofilm development in microfermenters (upper) or on the corresponding Pyrex slides removed from the microfermenters (lower) after 24 h of growing in TSB-gluc at 37°C is shown.

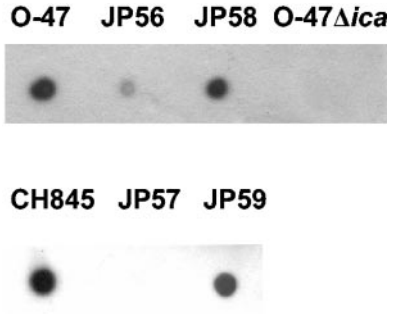


FIG. 4. Loss of PNAG production in *S. epidermidis sarA* mutant strains. Cell surface extracts from overnight cultures of *S. epidermidis* wild-type strains O-47 and CH845, their corresponding *sarA* mutants JP56 and JP57, respectively, the JP56 and JP57 strains complemented with plasmid pJP19 (strains JP58 and JP 59, respectively), and the O-47 Δ*ica*::*tet* as a negative control, treated as described in Materials and Methods, were spotted onto nitrocellulose filters. PNAG production was detected with an anti-PNAG polyclonal antibody. The *sarA* mutants produced lower levels of PNAG product.

biofilm. In contrast, JP56 only formed microcolonies on the surface of the slide and developed little biofilm thereafter.

Deletion of the *sarA* gene in *S. epidermidis* eliminates PIA/PNAG production. The impact of the SarA regulator on the production of PIA/PNAG, the product of the IcaADBC proteins, was initially investigated by a dot blot with a specific anti-PNAG polyclonal antiserum (gift from G. Pier). The specificity of the polyclonal antiserum was confirmed by the absence of signal with the *S. epidermidis* O-47 Δ*ica*::*tet* strain. Our results showed that the JP56 and JP57 *sarA* mutants produced decreased amounts of PNAG, whereas PNAG production was restored in the complemented mutants JP58 and JP59 (Fig. 4).

SarA upregulates *icaADBC* expression. The results discussed above suggested that *sarA* might control biofilm formation through *icaADBC* expression. To investigate whether the decrease in PIA/PNAG production observed in the *sarA* mutants was caused by a reduction of the *icaADBC* operon expression, we used real-time quantitative PCR. Although the extent of the decrease in the *icaA* RNA levels was different in both mutants, the results showed that the *sarA* mutation resulted in a significant ($P < 0.05$) decrease of *icaADBC* operon transcription compared to that of the wild-type strains at the mid-log exponential growth phase ($OD_{650} = 1$; Fig. 5). Similar results were obtained with RNA purified at the early stationary phase ($OD_{650} = 2$) (data not shown). These results indicate that SarA is a transcriptional activator of the *icaADBC* operon. However, it is worth noting the existence of considerable residual transcription of the *icaADBC* operon in the SarA mutant strain, a finding suggesting that SarA activity alone cannot account for the total *icaADBC* operon expression.

Recombinant SarA binds to the *icaA* promoter region. As the level of transcript of *icaA* was decreased in *sarA* mutants, we speculated that SarA may bind to the *icaA* promoter region to modulate *icaADBC* expression. To verify this, we employed a 200-bp *icaA* promoter (from *S. aureus* or *S. epidermidis*) for DNA binding assays. The DNA fragments were end labeled with [γ-^{32}P]ATP and used in gel shift assays with various amounts of purified SarA protein (Fig. 6A). The retarded protein-DNA complex could be detected with as little as 0.1 μg of SarA (≈3.3 nM). As the concentrations of the SarA protein increased, the retarded protein-DNA complex became the predominant band, with complete conversion at ≈0.2 to 0.3 μg of SarA. The presence of shifted bands with different sizes sug-

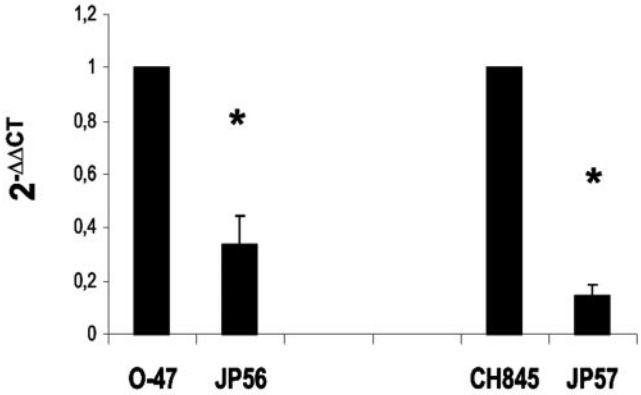
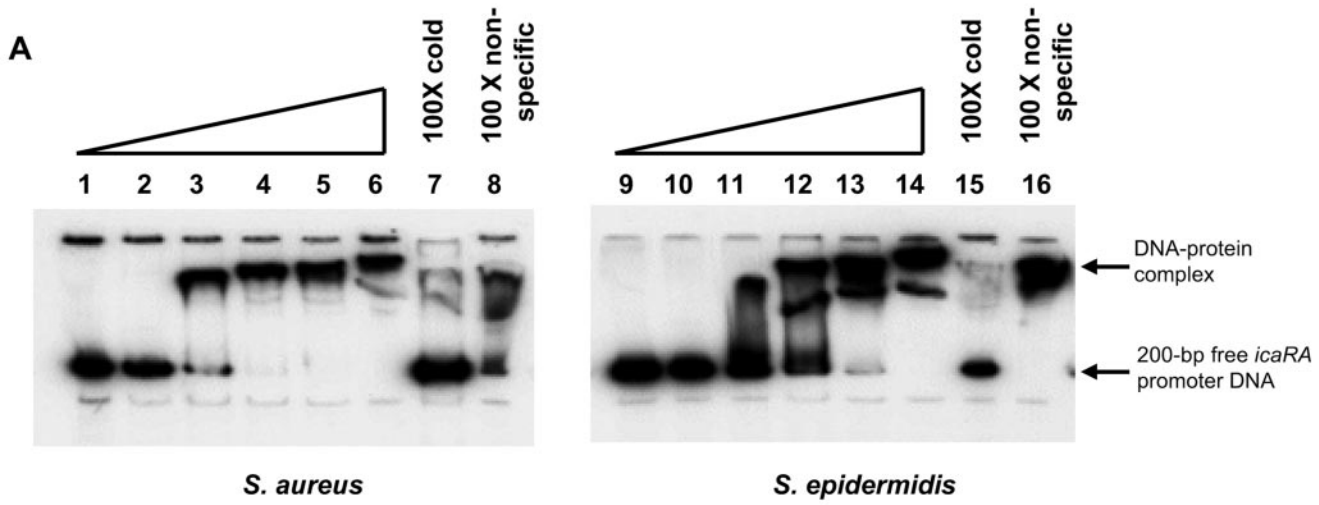


FIG. 5. Real time quantification of *ica* expression on *S. epidermidis* wild-type strains and their corresponding *sarA* mutants. Asterisks denote significance ($P < 0.05$).



B

***S. aureus* promoter region (AF086783)**

```
1
ATTGCGTTAT CAATAATCTT ATCCTTCAAT TTTTATAACC CCCTACTGAA AATTAATCAC ACTATGTTAC AGGAAAATTA
TAACGCAATA GTTATTAGAA TAGGAAGTTA AAAATATTGG GGGATGACTT TTAATTAGTG TGATACAATG TCCTTTTAAT
      icaR start codon                                                          Box I
81                                                    Box III
AGTTGCAATT ACAAATATTT CCGTTTAATT ATAACAACAA TCTATTGCAA ATTAAAATAC TATCAATTAC CATATGGCTT
TCAACGTTAA TGTTTATAAA GGCAAATTAA TATTGTTGTT AGATAACGTT TAATTTTATG ATAGTTAATG GTATACCGAA
      BoxI                                Box II
161                                                           Box IV
ACAACCTAAC TAACGAAAGG TAGGTAAAGA AATTGCAA ..icaA
TGTTGGATTG ATTGCTTTCC ATCCATTTCT TTAACGTT
```

***S. epidermidis* promoter region (U43366)**

```
1
ATTGCGTTAT CAATAATCTT ATCTTTCAAT TCTAAAATCT CCCCCTTATT CAATTTTCTA AAAATATATT ACAGAAAAAT
TAACGCAATA GTTATTAGAA TAGAAAGTTA AGATTTTAGA GGGGGAATAA GTTAAAAGAT TTTTATATAA TGTCTTTTTA
     icaR start codon                                                          Box I
81                                                    Box III
TAAGTTAAAA TTACAAATAT TACTGTTTCA GTATAACAAC ATTCTATTGC AAATTGAAAT ACTTTCGATT AGCATATGCT
ATTCAATTTT AATGTTTATA ATGACAAAGT CATATTGTTG TAAGATAACG TTTAACTTTA TGAAAGCTAA TCGTATACGA
        Box I                               Box II
161                                                           Box IV
TTACAACCTA ACTAACGAAA GGTAGGTGAA AAAATGCAT ..icaA
AATGTTGGAT TGATTGCTTT CCATCCACTT TTTTACGTA
```

FIG. 6. Autoradiogram of a nondenaturing 8% polyacrylamide gel with purified SarA protein and a 198-bp γ-^{32}P-radiolabeled DNA fragment containing the intergenic promoter region of the *icaRA* genes. A. Lanes 1 to 6, mobility of the 198-bp radiolabeled DNA fragment ($\approx$3 ng) of the *S. aureus icaRA* promoters region in the presence of 0, 50, 100, 200, 300, and 500 ng of purified protein, respectively; lanes 7 and 8, mobility of the same fragment with 300 ng of SarA, but in the presence of a 100-fold excess (molar ratio) of the unlabeled 198-bp fragment as the specific competitor (lane 7) and a 100-fold excess of unlabeled 148-bp intergenic *sarUT* promoter fragment (36) as the nonspecific competitor (lane 8). Lanes 9 to 16 are similar to lanes 1 to 8 except that the 198-bp fragment was from the *ica* promoter region of *S. epidermidis*. B. The nucleotide sequence of the 198-bp fragment containing the *ica* intergenic region of *S. aureus* (AF086783) and *S. epidermidis* (U43366) is shown and marked with the putative binding regions for SarA protein as determined based on the SarA consensus binding site (6).

gests the existence of several SarA-binding sites into the *icaADBC* promoter (Fig. 6B). Overall, these results support that SarA can bind to the *icaADBC* promoter and act as an activator of *icaADBC* transcription.

SarA does not affect *icaR* RNA levels. It has been reported that mutation of either σ^B or *rsbU*, an activator of σ^B, results in complete abolition of biofilm formation and a drastic decrease in *S. epidermidis ica* transcription (27, 28). A transcriptional analysis revealed that *icaR* is upregulated in these mutants lacking σ^B functions; apparently, a σ^B-dependent regulatory intermediate negatively regulates IcaR. It has also been shown that one of the *sarA* promoters (P1) is σ^B dependent (14, 27). We therefore speculated that σ^B might modulate *sarA* expression and the ensuing *ica* transcription. If such an association existed, expression of the *icaR* gene should be increased in the *sarA* mutants. An analysis of the transcriptional activity of the *icaR* gene by real-time PCR showed that the levels of *icaR* transcripts were not increased in the JP56 and JP57 *sarA* mutant strains (data not shown), a result suggesting control of the biofilm formation process by σ^B- and *icaR*-independent pathways.

DISCUSSION

Although the genetic arsenal responsible for pathogenesis differs between *S. aureus* and *S. epidermidis*, both species are capable of forming biofilms in a PIA/PNAG-dependent manner. In this context, a thorough comprehension of the mechanism by which PIA/PNAG is regulated in *S. epidermidis* and *S. aureus* is an important prerequisite for understanding biofilm formation and could ultimately lead to the development of methods to repress the expression of this important virulence factor. It is becoming increasingly apparent, however, that the transcriptional regulation of the genes involved in PIA/PNAG synthesis, the *ica* genes, is complex. This study provides experimental evidence to demonstrate that SarA regulates the expression of the *ica* operon in clinical isolates of *S. epidermidis*.

In *S. aureus*, the role of the SarA protein in pathogenesis has been extensively analyzed with regard to its involvement in the expression of extracellular and cell wall-associated virulence determinants. In addition, we and others have recently reported that SarA is essential for biofilm development in *S. aureus* (4, 47). However, little is known about the regulation of virulence by SarA in *S. epidermidis*. Without the typical virulence determinants of *S. aureus*, *S. epidermidis* is a common skin resident, the regulation of whose virulence determinants is still largely unknown. The existence of a SarA homolog in *S. epidermidis* encouraged us to examine whether SarA controls biofilm formation in coagulase-negative staphylococci. The discovery that SarA controls this process, intimately related to the persistence and antibiotic resistance of *S. epidermidis* infections, is a significant step in our initial approach toward understanding gene regulation in this pathogen.

Recently, in describing the role of σ^B in *S. epidermidis* biofilm formation, Knobloch and coworkers presented a model of the transcriptional and posttranscriptional regulation of PIA/PNAG synthesis and accumulation in that organism (27). This model revealed a complex regulation of PIA/PNAG synthesis, involving at least three different regulatory pathways. Two of these pathways act through the transcriptional regulation of the negative regulator IcaR, and the third pathway is a glucose-dependent proteinaceous factor of PIA/PNAG synthesis (12). Based on our results, we proposed two additional pathways: one depending on the global regulator SarA and the other depending on the δ-toxin, as previously described by Vuong and coworkers (48) (Fig. 7).

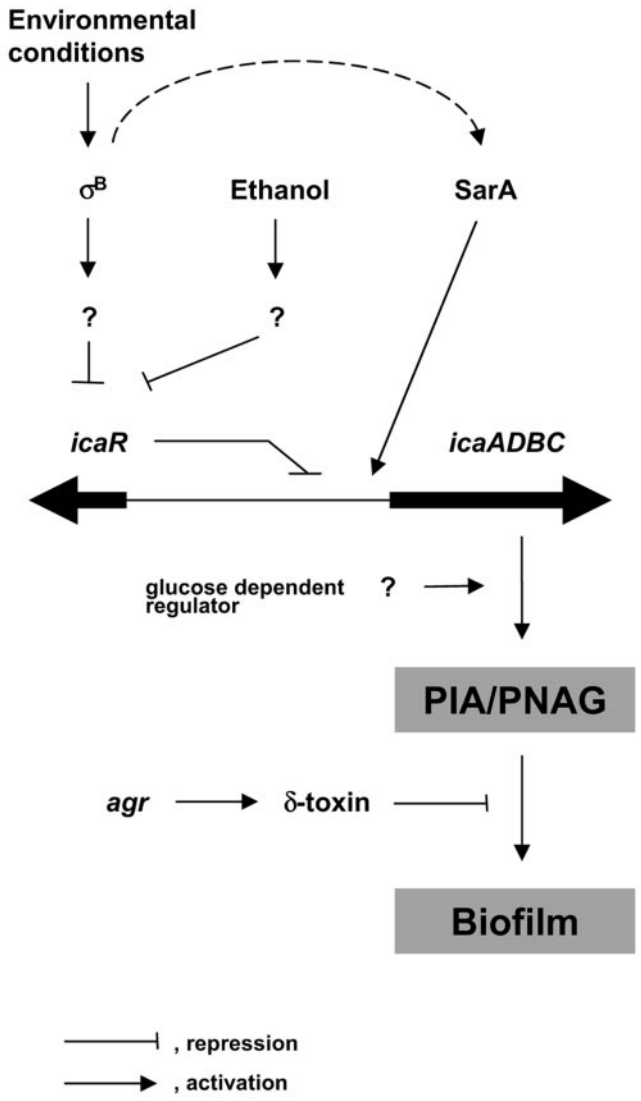


FIG. 7. Model of regulation of PIA/PNAG synthesis in *S. epidermidis*.

Two lines of evidences indicated that the pathway used by SarA in the control of *icaADBC* transcription is different from that utilized by σ^B. First, our results demonstrated that SarA binds to the *icaA* promoter and that *icaR* transcription is not increased in the SarA-defective strains in comparison to wild-type strains. In contrast, a σ^B deficiency provokes an upregulation of *icaR* transcription. Second, Knobloch and coworkers observed that supplementation of growth media with ethanol decreased *icaR* transcription in a σ^B mutant, a change leading to increased *icaA* transcription and a biofilm-positive phenotype. However, in these growth conditions, the σ^B-dependent *sarA* transcript was absent in the σ^B mutant, a finding suggest-

ing that SarA did not influence the IcaR-dependent regulation of PIA/PNAG synthesis (27). Furthermore, SarA influences the regulation of biofilm formation via an *agr*-dependent pathway.

The gene for δ-toxin is encoded within the gene for RNAIII, and its expression is therefore directly linked to *agr* activity (49). The *agr* two-component system does not affect the level of *icaADBC* expression. However, probably because of its detergent-like physicochemical properties, the *agr*-encoded δ-toxin, when present, abolished biofilm-forming capacity in *S. epidermidis* (48). Interestingly, although the *S. epidermidis* O-47 strain used in this study has been described as a natural *agr* mutant (48), deletion of the *sarA* gene in this strain inhibited biofilm development. These data are consistent with the conclusion that SarA affects biofilm formation via an *agr*-independent pathway.

In addition, other potential regulators remain to be included in this complex regulatory system. Two additional regulators, *tcaR* and *rbf*, present in the genomes of the sequenced *S. epidermidis* strains, have recently been described as being involved in *S. aureus* biofilm development. The *tcaR* gene is a negative regulator of *ica* transcription, though deletion of *tcaR* alone did not induce any changes in PIA/PNAG production or in adherence to polystyrene (25). On the other hand, Rbf is involved in the regulation of the multicellular aggregation step of *S. aureus* biofilm formation in response to glucose and salt. This regulation is probably mediated through a still-unidentified protein of 190 kDa (30). Finally, it is important to note that several SarA homologs, involved directly or indirectly in gene regulation, have been described in *S. aureus*. One of these regulators, SarR, has been identified in *S. epidermidis* (50), although is not present in all of the *S. epidermidis* strains analyzed (35).

In *S. aureus*, SarR, a 115-residue polypeptide, represses SarA expression during the postexponential phase by binding to the *sarA* promoter region (35). If the function of the SarR homolog in *S. epidermidis* is similar to that assigned in *S. aureus*, it is tempting to speculate that, in a mature biofilm colonizing medical devices, the expression of SarR by some bacteria could turn off their biofilm formation capacities, so that individual *S. epidermidis* cells could leave the biofilm and colonize new surfaces.

Proteolytic enzymes are secreted by a large number of prokaryotic organisms. In most cases, they are involved in nutrient acquisition, but a growing body of evidence indicates that peptidases produced by pathogenic bacteria are important virulence factors. In *S. aureus*, the expression of proteinases is tightly regulated at the level of transcription by the global regulators *agr*, *sarA*, and σ^B (46). In addition, proteolytic activity is controlled at the posttranslational level by a cascade of activation of the secreted zymogens. Both systems work in concert to regulate the function of these enzymes, which are suggested to facilitate *S. aureus* dissemination from initial colonization sites (32). This process occurs via an elaborate modification of bacterial surface proteins (26, 37, 38), changing the bacterial phenotype from adhesive to invasive.

Similar to *S. aureus*, *S. epidermidis* Δ*sarA* overproduces proteases. Evidence supports the implication of proteases in the regulation of biofilm development. Thus, the inactivation of *gelE*, encoding a zinc-metalloprotease gelatinase, prevents, by a still-uncharacterized mechanism, the primary attachment of and biofilm development by *Enterococcus faecalis* (21). Furthermore, it has been demonstrated that the regulation of the Hms phenotype of *Yersinia pestis*, involved in biofilm formation, results from the degradation of the HmsH, HmsR (homolog to IcaA), and HmsT proteins at 37°C (41).

In a previous study, we showed that inhibition or deletion of the main proteases of *S. aureus* (*aur* and *ssp*) in a *sarA*-null background was unable to restore biofilm formation. In this study, we used microfermenters in which the continuous replenishment of medium might impair the accumulation of high quantities of proteases in the growing extracellular medium. However, we cannot rule out the accumulation of a protease in the vicinity of the bacteria, a protease which, either by its physical presence or by its enzymatic activity, could be responsible for the biofilm deficiency of both the *S. epidermidis* and *S. aureus* strains. The cleavage of a surface protein could affect the hydrophobicity of the bacterial surface and prevent its attaching to surfaces such as plastic or glass. Alternatively, the overproduction of proteases could result in the degradation of Ica proteins responsible for PIA/PNAG synthesis. This rationale could explain the dissociation between residual *icaADBC* transcription and the absence of PIA/PNAG production in *sarA* mutants. In this context, it is important to note that glucose-dependent posttranscriptional regulation of PIA/PNAG synthesis has been described (12).

S. aureus and *S. epidermidis* are the gram-positive bacteria most often associated with medical implant-related infections. We have shown that both species control the PIA/PNAG-dependent biofilm formation process via *sarA* and that deletion of the *sarA* genes reduces the ability to produce PIA/PNAG and form a biofilm in vitro. Due to the high level of morbidity associated with *S. epidermidis* and *S. aureus* infections as well as the high frequency of infection by both organisms, the *sarA* gene could represent an important potential clinical target for the prevention of chronic infections associated with prosthetic medical devices.

ACKNOWLEDGMENTS

We express our gratitude to G. B. Pier for providing the polyclonal antibodies against PNAG; F. Götz for plasmids pCU1 and pSC20 and strains O-47 and O-47 Δ*ica::tet*; Névine El Solh for strain CH845 (BM94314); and M. Arnaud and M. Debarbouille for plasmid pMAD.

This work was supported by grant BIO2002-04542-C02-01 from the Comisión Interministerial de Ciencia y Tecnología (C.I.C.Y.T.) and grants from the Cardenal Herrera-CEU University, from the Conselleria d'Agricultura, Pesca i Alimentació, and from the Generalitat Valenciana (CTIDIA/2002/62) to J.R.P. A.L.C. acknowledges financial support provided by the NIH (grant AI37142). Fellowship support for María Ángeles Tormo from the Conselleria de Cultura, Educación y Deporte and for Miguel Martí from the Conselleria d'Agricultura, Pesca i Alimentació is gratefully acknowledged.

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