

## SarA Positively Controls Bap-Dependent Biofilm Formation in *Staphylococcus aureus*

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Received 13 May 2005/Accepted 24 May 2005

**The biofilm-associated protein Bap is a staphylococcal surface protein involved in biofilm formation. We investigated the influence of the global regulatory locus *sarA* on *bap* expression and Bap-dependent biofilm formation in three unrelated *Staphylococcus aureus* strains. The results showed that Bap-dependent biofilm formation was diminished in the *sarA* mutants by an *agr*-independent mechanism. Complementation studies using a *sarA* clone confirmed that the defect in biofilm formation was due to the *sarA* mutation. As expected, the diminished capacity to form biofilms in the *sarA* mutants correlated with the decreased presence of Bap in the bacterial surface. Using transcriptional fusion and Northern analysis data, we demonstrated that the *sarA* gene product acts as an activator of *bap* expression. Finally, the *bap* promoter was characterized and the transcriptional start point was mapped by the rapid amplification of cDNA ends technique. As expected, we showed that purified SarA protein binds specifically to the *bap* promoter, as determined by gel shift and DNase I footprinting assays. Based on the previous studies of others as well as our work demonstrating the role for SarA in *icaADBC* and *bap* expression (J. Valle, A. Toledo-Arana, C. Berasain, J. M. Ghigo, B. Amorena, J. R. Penades, and I. Lasa, *Mol. Microbiol.* 48:1075–1087), we propose that SarA is an essential regulator controlling biofilm formation in *S. aureus*.**

Biofilm formation is a major concern in nosocomial infections because it protects microorganisms from opsonophagocytosis and antibiotics, leading to chronic infection and sepsis (13). To date, two surface components have been implicated in biofilm formation by *Staphylococcus aureus*: (i) the product of the *icaADBC* operon, which encodes proteins for the synthesis of the polysaccharide poly-*N*-acetyl  $\beta$ -1-6-glucosamine (PNAG) (14, 26), and (ii) Bap, a surface protein that is essential for biofilm formation in some bovine *S. aureus* strains (15). Although the *bap* gene was only present in a small fraction of bovine mastitis isolates (5%) and was absent from the 75 clinical human *S. aureus* isolates analyzed, all the staphylococcal isolates harboring *bap* were highly adherent and strong biofilm producers (15). The *bap* gene, carried in a putative composite transposon inserted in a mobile staphylococcal pathogenicity island (40), codes for Bap, a multidomain protein with architecture characteristic of surface-associated proteins from gram-positive bacteria (19). Experiments carried out in vitro have shown that Bap promotes early adherence and intercellular adhesion of *S. aureus* cells (15). Experimental mammary gland infection studies have indicated that Bap might act as an antiattachment factor that prevents initial bacterial attachment to host tissues (16), although Bap-positive isolates were significantly more able to persist in the bovine mammary gland in

vivo and were less susceptible to antibiotic treatments when forming biofilms in vitro (17).

An evaluation of the biofilm formation process of *S. aureus* will likely contribute to our understanding of the infectious process. Temporal expression of many of the virulence determinants in *S. aureus* has been shown to be under the control of several genetic loci, including *agr* and *sarA*. The *agr* locus is a complex multigene system that regulates virulence genes in response to increasing cell density (for a review, see reference 30). The *sarA* locus codes for SarA, a 14.5-kDa DNA-binding protein that activates *agr* promoters and thus can work in concert with the *agr* system to control target gene transcription (9, 11). The SarA protein can also activate some virulence genes independently of *agr* (7, 44). SarA binds to a consensus motif upstream of the  $-35$  sequences of the promoters of SarA-dependent genes (12).

Previously, SarA was shown to be a positive regulator of *S. aureus* PNAG-dependent biofilm formation (4, 41). Our results demonstrated that nonpolar mutations of *sarA* in four genetically unrelated *S. aureus* strains decreased PNAG production and completely impaired biofilm development, both under steady-state and flow conditions, via an *agr*-independent mechanism. Real-time PCR showed that the mutation in the *sarA* gene resulted in downregulation of *ica* operon transcription (41). Here we analyzed the role of SarA in expression of Bap, the other surface component involved in *S. aureus* biofilm formation. As described for the *icaADBC* operon, the *bap* gene is positively regulated by SarA by an *agr*-independent mechanism. Taken together, these results suggest that *sarA* is an important regulator of the biofilm formation process of *S. aureus*.

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TABLE 1. Bacterial strains used in this study

Strain or plasmid	Relevant property(ies)	Reference
<i>S. aureus</i> strains		
V329	Bovine clinical isolate, <i>bap</i> positive	22
c104	Bovine clinical isolate, <i>bap</i> positive	40
V858	Bovine clinical isolate, <i>bap</i> positive	This study
RN4220	Restriction-deficient mutant of 8325-4	24
CYL316	Recipient strain for plasmid pCL84	25
SH1000	Functional <i>rsbU</i> derivative of 8325-4	22
Newman	Natural isolate	18
PC1839	8325-4 <i>sarA::km</i>	8
ISP546	8325-4 <i>agr::Tn551</i>	32
AK1	8325-4 <i>aur::ermB</i>	23
AK2	8325-4 <i>ssp::ermB</i>	23
JP60	Newman with the <i>bap</i> gene from the V858 strain inserted in its genome	This study
JP61	SH1000 with the <i>bap</i> gene from the V858 strain inserted in its genome	This study
JP62	V329 $\Delta$ <i>sarA</i>	41
JP63	c104 $\Delta$ <i>sarA</i>	This study
JP64	JP60 <i>sarA::km</i>	This study
JP65	JP61 <i>sarA::km</i>	This study
JP66	JP62 pCU1- <i>sarA</i>	This study
JP67	JP63 pCU1- <i>sarA</i>	This study
JP68	JP64 pCU1- <i>sarA</i>	This study
JP69	JP65 pCU1- <i>sarA</i>	This study
JP70	JP62 pCU1	This study
JP71	JP63 pCU1	This study
JP72	JP64 pCU1	This study
JP73	JP65 pCU1	This study
JP74	JP64 <i>aur::ermB</i>	This study
JP75	JP64 <i>ssp::ermB</i>	This study
JP76	JP65 <i>aur::ermB</i>	This study
JP77	JP65 <i>ssp::ermB</i>	This study
JP78	JP61 pJP21	This study
JP79	JP65 pJP21	This study
JP80	V329 $\Delta$ <i>agr</i>	41
JP81	JP60 <i>agr::ermB</i>	This study
JP82	JP61 <i>agr::ermB</i>	This study
<i>E. coli</i> strains		
DH5 $\alpha$	Laboratory strain	
Plasmids		
pCL84	Integrating plasmid	25
pJP17	pCL84 carrying the <i>bap</i> gene under the the control of its native promoter	This study
pALC2485	Plasmid for construction of <i>luxABCDE</i> fusions	A. L. Cheung
pJP21	<i>bap</i> promoter- <i>luxABCDE</i> fusion formed by cloning the <i>bap</i> promoter region in plasmid pALC2485	This study
pCU1	Shuttle plasmid	2
pCU1- <i>sarA</i>	pCU1 carrying <i>sarA</i>	41

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. Phage 85 was used to transduce plasmids and mutations between staphylococcal strains (31).

Staphylococcal strains were cultured in trypticase soy agar (TSA) and in trypticase soy broth (TSB) supplemented with glucose (0.25%, wt/vol) when indicated. Media were supplemented when appropriate with chloramphenicol (10  $\mu$ g/ml for plasmids pCU1 and pJP21), erythromycin (2.5  $\mu$ g/ml for plasmid pMAD), or tetracycline (3  $\mu$ g/ml for plasmid pCL84).

**DNA manipulations.** Routine DNA manipulations were performed using standard procedures (3, 36). Plasmid DNA from *Escherichia coli* and staphylococci was purified with a Genelute Plasmid Miniprep kit (Sigma) according to the manufacturer's protocol, except that the staphylococcal bacterial cells were lysed with lysostaphin (Sigma; 12.5  $\mu$ g/ml) at 37°C for 1 h before plasmid purification. Plasmids were introduced into staphylococci strains by transformation using a previously described method (15). Restriction enzymes were purchased from Roche and used according to the manufacturer's instructions. Oligonucleotides were obtained from Invitrogen.

For Southern blot hybridization, staphylococcal chromosomal DNA was extracted using 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  $\mu$ g/ml) at 37°C for 1 h before DNA purification. DNA fragments were transferred by alkaline capillary blotting onto nylon membranes (Hybond-N; 0.45 mm pore-size filters; Amersham Life Science) using standard methods (3, 36). Probe labeling and DNA hybridization were performed according to the protocol supplied with the PCR-digoxigenin DNA-labeling and chemiluminescence detection kit (Roche).

**Biofilm formation.** Quantification of biofilm formation on abiotic surfaces was assessed basically as described elsewhere (21). Briefly, *S. aureus* was grown overnight in TSB supplemented with 0.25% glucose (TSB-glucose). The culture was diluted 1:40 in TSB-glucose, and 200  $\mu$ l of this cell suspension was used per well to inoculate sterile, 96-well polystyrene microtiter plates (Iwaki). After 18 h of incubation at 37°C, wells were gently washed three times with 200  $\mu$ l of sterile phosphate-buffered saline (PBS), air dried in an inverted position, and stained with 0.1% safranin for 30 s. Wells were rinsed again, and the absorbance was determined at 490 nm (Micro-ELISA Autoreader; Elx800 Bio-tek Instruments). Each assay was performed in triplicate in five separate experiments.

Colony morphology was studied on Congo red agar as previously described (15). A positive result indicating biofilm formation was demonstrated by the presence of black or pink colonies with a dry crystalline surface (rough colony

phenotype). Deficiency in biofilm formation was indicated by the presence of smooth colonies.

**Construction of *S. aureus* strains carrying a chromosomal copy of the *bap* gene.** A single-copy integrating plasmid, pCL84 (25), was used to introduce the *bap* gene into *S. aureus* strains Newman (ATCC 25905) and SH1000. Plasmid pCL84 carries the *att* site of phage L54a but lacks a replicon that functions in *S. aureus*. When transformed into *S. aureus* CYL316, which overexpresses the L54a integrase, the plasmid integrated into the chromosomal *att* site located in the *geh* gene (25). As previously described, the *bap* gene of the *S. aureus* V858 strain, including its native promoter, was amplified with Pfu DNA polymerase (Promega) by using primers Bap-1mB (5'-CGCGGATCCCTCTTCAGATCTACG AATTTTCCC-3') and Bap-5cE (5'-CGGAATTCACCTTATAGATGTGCGT AGTC-3') and was cloned in pCL84, generating pJP17 (17). Plasmid pJP17 was transformed into CYL316 and integrated into the chromosome by homologous recombination at the phage L54a *att* site. The *bap* gene was then transduced by phage 85 (31) into strains Newman and SH1000, generating strains JP60 and JP61, respectively. Correct integration of pJP17 in CYL316 and in JP60 and JP61 was verified by PCR and Southern blotting with lipase- and *bap*-specific probes.

**Allelic exchange of chromosomal genes.** The *sarA*, *aur*, *ssp*, and *agr* genes were inactivated in *S. aureus* Newman and SH1000 strains by transferring the *sarA*, *aur*, *ssp*, and *agr* mutations via phage transduction using  $\phi$ 85 (31). A strain c104 *sarA* mutant was obtained basically as described elsewhere (41).

**Complementation studies.** To prove that the biofilm-deficient phenotype of the mutants was due to the disruption of *sarA*, mutant strains were complemented with plasmids pCU1 (2) or pCU1-*sarA*. Plasmid pCU1-*sarA* carries the *sarA* gene under the control of its promoter cloned in pCU1 (41). Plasmids pCU1 and pCU1-*sarA* were transformed into strain RN4220 by electroporation (15). Phage 85 was used to transduce the plasmids from RN4220 to *sarA* mutant strains.

**Western blot analysis and zymography.** The Bap immunoblotting assay was performed as described previously (16). Briefly, *S. aureus* cells from a stationary-phase culture were resuspended to an optical density at 600 nm ( $OD_{600}$ ) of 40 in 100 mM PBS containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Cells were centrifuged and suspended in 1 ml of digestion buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, and 30% raffinose [Sigma]). To each 1-ml sample, 60  $\mu$ l protease inhibitors (Complete cocktail, Boehringer Mannheim), 40  $\mu$ l 50 mM phenylmethylsulfonyl fluoride, and 60  $\mu$ l of a 2-mg/ml solution of lysozyme (Sigma) were then added, and the suspension was incubated in a 37°C water bath for 30 min. Protoplasts were sedimented by centrifugation at 6,000  $\times$  g and the supernatant fraction, which contained the wall-associated protein, was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel, 4.5% stacking gel).

For Western blot analysis, protein extracts analyzed by SDS-PAGE as described above were blotted onto Immobilon P membrane (Millipore). Anti-Bap serum (15) was diluted 1:2,500 with TTBS (Tris-buffer saline [50 mM Tris-HCl, pH 7.5, 150 mM NaCl] containing 0.05% Tween 20) and immunoadsorbed with 1% skimmed milk. Alkaline phosphatase-conjugated protein A (Sigma) diluted 1:10,000 in TTBS-1% skimmed milk was used, and the subsequent chemiluminescence reaction (CSPD; Roche) was recorded.

Zymogram analysis was performed as previously described (35). Briefly, culture supernatants were subjected to SDS-PAGE using 12% acrylamide gels containing gelatin (1 mg/ml; Difco). Following electrophoresis, the gels were shaken gently for 60 min at room temperature in phosphate-buffered saline (PBS) containing 2.5% (vol/vol) Triton X-100 (Sigma). The gels were then incubated overnight at 37°C in buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.02% (vol/vol) Triton X-100, and 1 mM cysteine. The gels were then stained with Coomassie blue dye and destained to reveal zones of protease activity.

**Isolation of RNA and Northern blot hybridization.** Total RNA from *S. aureus* was prepared by using a TRIzol isolation kit (Gibco BRL, Gaithersburg, Md.) and a reciprocating shaker as described elsewhere (27). The optical density at 650 nm ( $OD_{650}$ ) of various cultures was determined with a spectrophotometer (Spectronic 20). The concentration of RNA was determined by measuring the absorbance at 260 nm, and 10  $\mu$ g of total RNA was analyzed by Northern blotting as described previously (27). As probe, a fragment of the *bap* gene was amplified by PCR using oligonucleotides *sasp*-6m (5'-CCCTATATCGAAGGTGTAGAATTGCAC-3') and *sasp*-7c (5'-GCTGTTGAAGTAACTACTGTACC TGC-3'). An internal fragment of the 16S rRNA gene (nucleotides 777 to 1500; GenBank accession no. X68417; oligonucleotides 16S rRNA-r, 5'-CCCCAATC ATTTGTCCACC-3', and 16S rRNA-f, 5'-GCGTGGGGATCAACAGG-3') was used as a loading control. For detection of specific transcripts, gel-purified DNA probes were radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the random-primed DNA labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-

phase conditions at 65°C. The blots were subsequently washed and autoradiographed.

**Transcriptional fusion of the *bap* promoter to the *luxABCDE* reporter genes.** The promoter region and the ribosome-binding site from the *bap* gene was PCR amplified using oligonucleotides pbap3mE (5'-CGGAATTCGAGGTAGTTA CAGATCAGGCACCT-3') and pbap-2cA (5'-CCCCGGGGAAAATAATT TTTTACAATTTTATGACGC) and cloned in the EcoRI-AvaI sites of plasmid pALC2485, a derivative of pSK236 containing *luxABCDE* at the Sall/PstI site, generating pJP21. Plasmid pJP21 contains the transcriptional fusion of the *bap* promoter to the *luxABCDE* reporter genes. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the constructs. The recombinant plasmid was first electroporated into *S. aureus* strain RN4220. Plasmid from RN4220 was then transferred by phage transduction into JP61 and JP65, the parental strain SH1000, and its *sarA* mutant containing the *bap* gene, respectively, generating JP78 and JP79. Bioluminescence in these strains was detected in a luminometer (Lumimark; Bio-Rad). Briefly, overnight cultures were diluted (1:100) and grown at 37°C. One-hundred microliters of the sample in triplicate was then withdrawn from the culture and assayed in microtiter plates (catalog no. 3632; Costar). The data were reported as bioluminescence unit/optical density ( $OD_{650}$ ).

**Purification of SarA protein.** The cloning and purification of the His<sub>6</sub>-tagged fusion SarA protein were described earlier (12). The purity of the purified His<sub>6</sub>-tagged SarA fusion protein was confirmed by sodium dodecyl sulfate (SDS) gels stained with Coomassie Brilliant Blue R-250. The purified His<sub>6</sub>-tagged SarA protein was found to be more than 98% pure in an SDS-12% polyacrylamide gel. The concentration of the purified proteins was determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.), using bovine serum albumin as the standard.

**Gel shift analysis and DNase I footprinting.** To determine if the SarA protein binds to the *bap* promoter region, a 228-bp PCR-amplified fragment (oligonucleotides Bap-1mB and pbap-2cA), representing the *bap* promoter region, was end labeled with [ $\gamma$ -<sup>32</sup>P]ATP by using T4 polynucleotide kinase. Labeled DNA fragment (0.1 ng or 0.5 fmol) was incubated at room temperature (RT) for 20 min with various amounts of purified SarA protein in 25  $\mu$ l 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  $\mu$ g 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.

Footprinting assays with template DNA fragment and DNase I were performed as previously described (3, 12). Upper and lower primers used for amplifying the promoter region were 5'-ATACGGCAAAGAATACCTTTAAAA G-3' and 5'-AAATAAATTTTTTACAATTTTATGACGCA-3', respectively. To label PCR products, only one of the primers was labeled at one end. For the assay, the binding reactions were carried out in a 100- $\mu$ l reaction volume containing 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM dithiothreitol, 10  $\mu$ g of bovine serum albumin, 0.4  $\mu$ g of calf thymus DNA, radiolabeled template DNA (20,000 cpm), and various amounts of the purified SarA protein at RT for 30 min. DNase I (0.02 U; Boehringer, Mannheim, Germany) was added and allowed to incubate for 1 min at RT. The reaction mixtures were extracted with phenol-chloroform, ethanol precipitated, washed with 70% ethanol, dried, and resuspended in loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.025% [wt/vol] xylene cyanol FF, 0.025% [wt/vol] bromophenol blue). DNA samples were denatured at 95°C for 5 min and analyzed on a 6% denaturing polyacrylamide sequence gel. The positions of the protected region were derived by comparing the footprint with the A+G sequencing ladder of the same fragment.

**5'-RACE (rapid amplification of cDNA 5' ends).** Amplification of the *bap* cDNA 5' end was performed using the 5'/3' RACE kit (Roche), according to the manufacturer's protocol. First-strand cDNA synthesis was performed using the oligonucleotide *bap*5SP1-c (5'-TGGTAGATGCATCTTCATCTATTGC-3') according to the manufacturer's instructions. The cDNA mixtures were amplified by PCR using the oligo(dT) anchor primer and the gene-specific primer *bap*5SP2-c (5'-CAGAAGATTGTGATGATGTATTTCG-3'). The obtained PCR products were analyzed in 1.2% agarose gels.

**Statistical analysis.** The data were analyzed by Student's *t* test for unpaired data to determine statistically significant differences. Differences were considered statistically significant when *P* was <0.05 in all cases.

## RESULTS

**Construction and characterization of different derivatives of laboratory strains carrying the *bap* gene.** In a previous study,

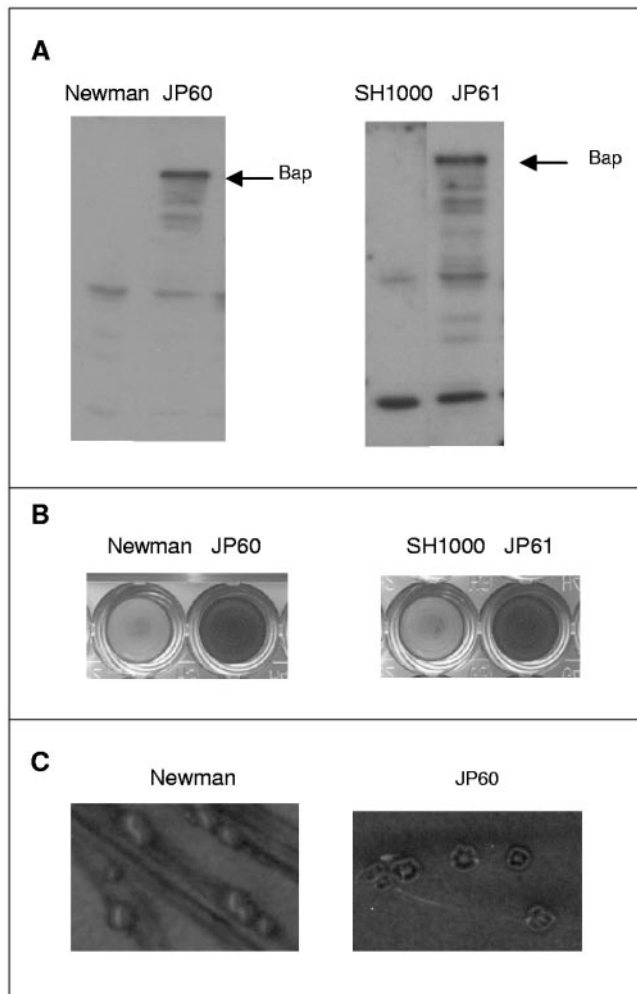


FIG. 1. A. Study of the presence of Bap by Western blotting. An approximately 140-kDa band is recognized by polyclonal antibodies against Bap only in *bap*-integrated strains JP60 and JP61. B. Biofilm formation phenotype. Significant differences ( $P < 0.05$ ) between the wild-type strains Newman and SH1000 and the *bap*-integrated JP60 and JP61 strains in the capacity to form a 16-h biofilm on polystyrene microtiter plates after staining with safranin was observed. C. Phenotypic differences between the wild-type strain Newman and the *bap*-complemented strain JP60 in Congo red agar colony morphology.

we showed that a mutation in the *sarA* gene abolished biofilm formation in V329, the prototypical *bap*-positive strain (41). Considering that biofilm formation in this strain is Bap dependent (17), our result implies that expression of the Bap protein could be regulated by SarA. To confirm that *sarA* regulates *bap* gene expression, we first constructed JP60 and JP61, two recombinant derivative strains of Newman and SH1000, respectively, in which the *bap* gene had been integrated into the bacterial chromosome. We constructed these strains in these backgrounds because of their ease of genetic manipulation. In addition, both of these strains contain functional global regulators, in contrast to some clinical isolates, in which some regulators are muted. We observed that expression of the Bap protein in JP60 and JP61 strains (Fig. 1A) rendered the bacteria competent for biofilm formation on microtiter wells (Fig.

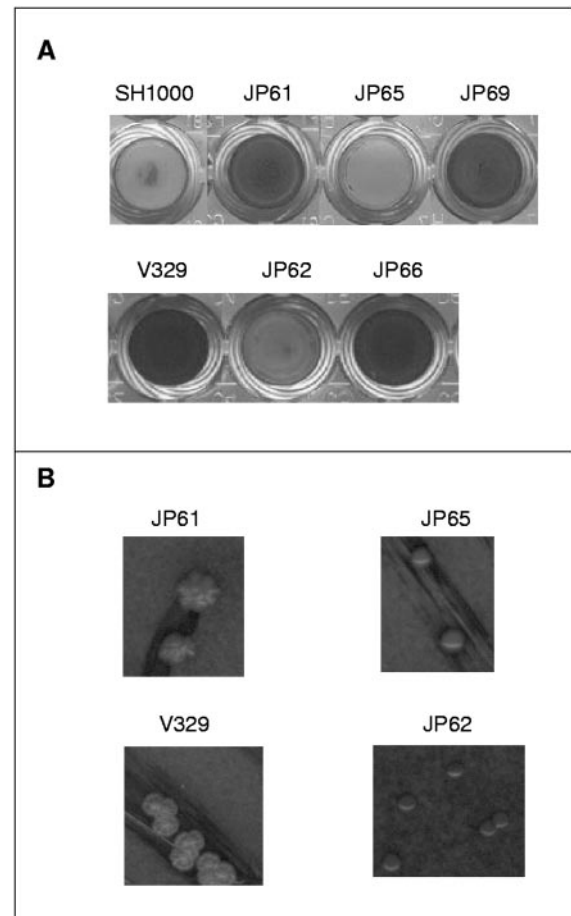


FIG. 2. A. Analysis of biofilm formation by *S. aureus* JP61 and V329, their respective *sarA* mutants JP65 and JP62, and their respective *sarA* mutants complemented with plasmid pCU1-*sar* (strains JP69 and JP66). Significant differences in adherence ( $P < 0.05$ ) were found between wild-type strains and their respective *sarA* mutants, as well as the *sarA* mutants versus *sarA* mutants complemented with pCU1-*sarA*. B. Phenotypic differences between Bap-expressing strains JP61 and V329 and their *sarA* mutants in Congo red agar colony morphology.

1B). In contrast to the parental strains, Bap-expressing strains were positive for Congo red staining (Fig. 1C).

**Effect of *sarA* on production of Bap and biofilm formation.** Null *sarA* mutants of *S. aureus* strains V329 and c104 (wild-type strains) and JP60 and JP61 (recombinant strains) were analyzed for their ability to form biofilm on microtiter wells in polystyrene plates. The *sarA* mutant strains JP62 (derivative of V329) and JP65 (derivative of JP61) were deficient in biofilm formation (Fig. 2A) and formed smooth colonies on Congo red agar plates (Fig. 2B). Similar results were obtained when strains JP63 (c104 *sarA* mutant) and JP64 (JP60 *sarA* mutant) were analyzed (data not shown).

To confirm that the biofilm-deficient phenotypes were due to *sarA* mutations, plasmid pCU1-*sarA* (carrying a PCR-amplified fragment containing the *sarA* gene under the control of its promoter) was introduced into the *sarA* mutants JP62, JP63, JP64, and JP65, generating strains JP66 to JP69, respectively. The vector control was also introduced into the above *sarA* mutant strains, generating strains JP70 to JP73. As shown in

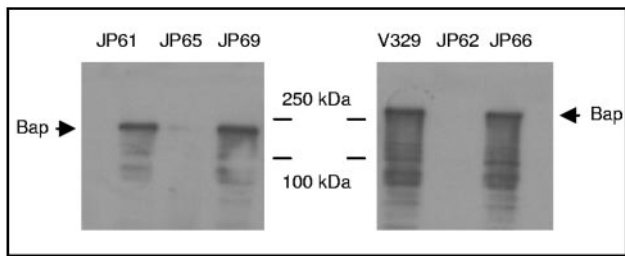


FIG. 3. Western blot analysis of Bap protein in *S. aureus* strains JP61 and V329, their respective *sarA* mutants JP65 and JP62, and the *sarA* mutants complemented with plasmid pCU1-*sarA* (JP69 and JP66). Bap protein production was detected with an anti-Bap antiserum.

Fig. 2A, the complemented strains JP69 and JP66 displayed a biofilm phenotype similar to that of the wild-type strains. Similar results were obtained when we analyzed the complemented strains JP67 and JP68 (data not shown).

**Effect of *sarA* on the expression of biofilm-associated protein.** The expression of Bap in V329 and JP61 and their respective *sarA* mutant strains was also analyzed by Western blotting. Consistent with the results of biofilm formation, the cell wall extracts of the *sarA* mutants displayed undetectable Bap levels compared to the respective isogenic parents (Fig. 3). The expression of the Bap protein was restored in the complemented strains JP69 and JP66 (Fig. 3). As previously described, the size of the Bap protein varies between strains due to the number of the C-repeats present in the protein (17).

**The relationship between the extracellular proteolytic activity and the biofilm formation capacity of the *sarA* mutant strains.** Several extracellular proteases are overexpressed in the *sarA* mutants (8). To determine if proteolytic cleavage of the cell-wall-associated Bap protein resulted in loss of biofilm-forming capacity in *sarA* mutants, we first constructed different *sarA* mutants harboring a deletion of the aureolysin gene (*aur*) or the serine protease gene (*ssp*) and then determined the ability of the double mutants to produce a biofilm. The JP74 (derivative of JP64; *aur* mutant), JP75 (derivative of JP64; *ssp* mutant), JP76 (derivative of JP65; *aur* mutant), and JP77 (derivative of JP65; *ssp* mutant) strains displayed a reduced capacity to form biofilm, similar to that of their isogenic *sarA* mutant strains (Fig. 4A), while their capacities to produce proteases were diminished, as verified by zymographic analysis (Fig. 4B) and by growing the mutants in casein agar plates (Fig. 4C).

In addition, we performed biofilm formation assays on microtiter plates with the wild type and *sarA* mutants grown in the presence of  $\alpha_2$ -macroglobulin, a universal protease inhibitor that inhibits the activity of all major staphylococcal proteases (41), and E64, a cysteine protease inhibitor, that inhibits two of the major *S. aureus* proteases (SspB and Scp). No significant differences were found in the biofilm-forming capacity of *sarA* mutant cells grown in the presence or absence of  $\alpha_2$ -macroglobulin or E64 (data not shown). These data suggest that extracellular proteases were likely not responsible for the biofilm deficiency of *sarA* mutant cells.

**Transcriptional analysis of *bap* gene expression in *sarA*-deficient strains.** To determine whether the effect of *sarA*

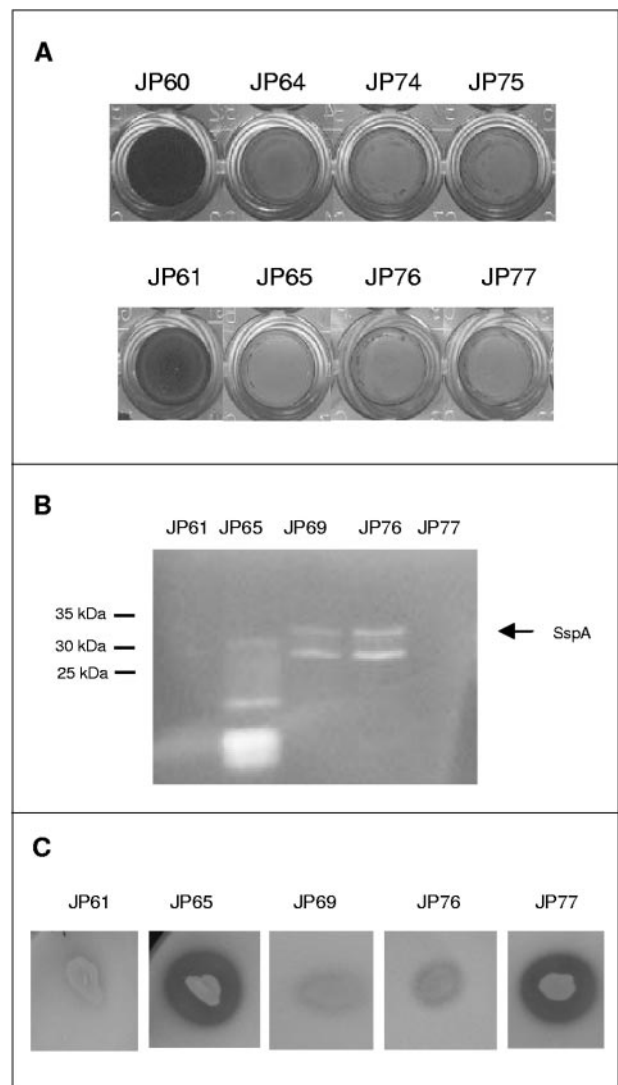


FIG. 4. (A) Comparison of the ability to produce a biofilm on polystyrene microtiter dishes of *S. aureus* *bap*-integrated strains (JP60 and JP61), their corresponding *sarA* mutants (JP64, JP65), and the *sarA-aur* and *sarA-ssp* mutant strains (JP74 and JP75 as well as JP76 and JP77, respectively). (B) Zymogram detection of secreted protease activity after electrophoresis in 12% acrylamide gels containing gelatin as substrate. Protease activity appears as a clear zone against a Coomassie blue-stained background. (C) Protease production by *S. aureus* strains in casein agar plates.

occurs at the transcriptional level, expression of the *bap* gene during the growth cycle of JP78 (JP61 carrying pJP21, the *bap* promoter *luxABCDE* reporter construct) and its *sarA* mutant JP79 (JP65 carrying pJP21) was monitored with a transcriptional fusion in which the *bap* promoter drives expression of *luxABCDE*. For strain JP78, maximum *bap* expression was observed during the exponential phase of growth (Fig. 5A). The *sarA* mutant strain JP79 showed minimal transcription of the *bap* gene during the entire growth cycle (Fig. 5A).

To confirm that *sarA* controls *bap* expression at the transcriptional level, we performed Northern blot analysis. Samples from JP60, JP61, JP64, and JP65 cultures were processed for total RNA preparation at different time points. The RNAs

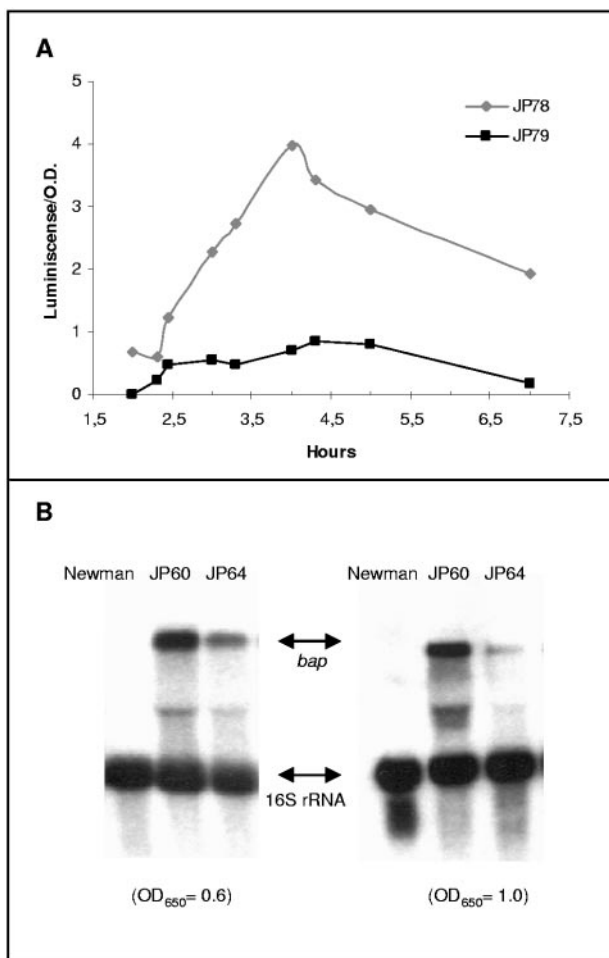


FIG. 5. (A) Transcription of *bap* detected with a *bap::luxABCDE* reporter fusion. Transcription of *bap* was monitored throughout the growth cycle of strain JP78 (JP61 carrying pJP21) and its *sarA* mutant JP79 (JP65 carrying pJP21). (B) Comparative measurement by Northern analysis of *bap* and 16S rRNA (control) transcription in wild-type *bap*-integrated JP60 and *sarA* mutant JP64 *S. aureus* strains. RNA was prepared from cultures grown in TSB at 37°C to early ( $OD_{650} = 0.6$ ) or late exponential phase ( $OD_{650} = 1$ ) of the growth curve. Expression of 16S rRNA is constitutive and was used as an internal control of this experiment.

were probed with a *bap* gene probe and with a 16S rRNA probe to monitor sample loading. An example of the Northern blot is shown in Fig. 5B. Similar results were obtained with samples from JP61 and JP65 (data not shown). As previously described, the expression of the *bap* gene was reduced in the *sarA* mutant strain.

**SarA controls Bap expression by an agr-independent mechanism.** Considering that SarA is a positive regulator of the *agr* operon, and that *agr* regulates various virulence phenotypes including biofilm formation (38), it is conceivable that SarA could affect biofilm formation indirectly via *agr*. However, we had demonstrated previously that strain JP80 (*agr* mutant derivative of V329) was not affected in its capacity to form a biofilm (40), suggesting that SarA affected *bap* expression via an *agr*-independent pathway. To test this hypothesis, we created insertional *agr* mutations in JP60 and JP61 strains, gen-

erating strains JP81 and JP82, respectively. Neither Bap expression nor biofilm formation capacity in microtiter wells was affected in the *agr* mutant strains (data not shown).

**Transcriptional start sites and promoter structure of the *bap* gene.** To determine the transcriptional start site and the promoter sequence, 5'-RACE analysis was performed with total RNA isolated from the wild-type strains V329 and JP61. The transcriptional start site was mapped to a G, which was located 106 bp upstream of the initiation codon ATG (Fig. 6C). Based upon the transcriptional start site, the predicted putative promoter boxes are TTTACT(-35)-N<sub>16</sub>-TATAAT(-10), which has close homology with the -10 and -35 consensus sequences of  $\sigma^A$ -dependent promoters (Fig. 6B). A ribosome binding site, GAGGTG, was located 7 bp upstream of the ATG translational start codon.

**SarA binds to the *bap* promoter.** Because *sarA* controls *bap* expression by an *agr*-independent mechanism we tested whether SarA binds specifically to the *bap* promoter. Histidine-tagged SarA was expressed in *Escherichia coli* and purified to homogeneity. Purified recombinant SarA was allowed to bind to an end-labeled 228-bp PCR fragment representing the entire *bap* promoter in electrophoretic mobility shifts assays. As shown in Fig. 7, the recombinant SarA protein induced shifts in the *bap* promoter probe. The binding appeared to be specific because SarA-induced shifts were not affected when a 10-fold excess of nonspecific unlabeled DNA fragment was used as a competitor (Fig. 7). Hence, SarA protein can bind to the *bap* promoter region, presumably acting as an activator to *bap* transcription. To verify the DNA binding specificity and to map the binding sites, we performed DNase I protection assays with a 295-bp *bap* promoter fragment labeled with  $\gamma$ -<sup>32</sup>P at one end in the presence of SarA. As shown in Fig. 6, protection from DNase I digestion with SarA was mapped to three regions very close to each other. The putative protected regions of SarA are from positions -169 to -146 bp (Fig. 6, top strand of DNA) and -55 to -30 and -19 to +61 bp of the transcriptional start site (Fig. 6, bottom strand of DNA). A close analysis of this sequence revealed that the protected regions closely resemble the SarA consensus DNA binding motif (ATTTGT ATTTAATATTTATATAATTG) previously reported (12). Boxes I, II, and III have 15 of 25, 17 of 25, and 15 of 25 matches to the SarA consensus binding sequence, respectively, thus verifying that the DNA binding site of SarA on the *bap* promoter is likely specific and further confirming previous studies on the predicted SarA binding consensus sequence.

**DISCUSSION**

Although PNAG, the product of the intercellular adhesion locus (*icaADBC*), has been shown to be critical for biofilm formation in *S. aureus*, it has been recently reported that *ica* is not absolutely required for biofilm formation in at least some strains of *S. aureus*. This finding corroborated our recent data, showing that *bap*-positive strains are capable of biofilm formation in the absence of the *ica* operon (17). In addition, Beenken et al. demonstrated that mutation of *ica* in strain UAMS-1 had little impact on biofilm formation (5). In this study, we evaluated the regulation of the factor Bap by the global regulators *agr* and *sarA*. Examination of *bap* mRNA production by different strains at various growth phases showed that *sarA* was

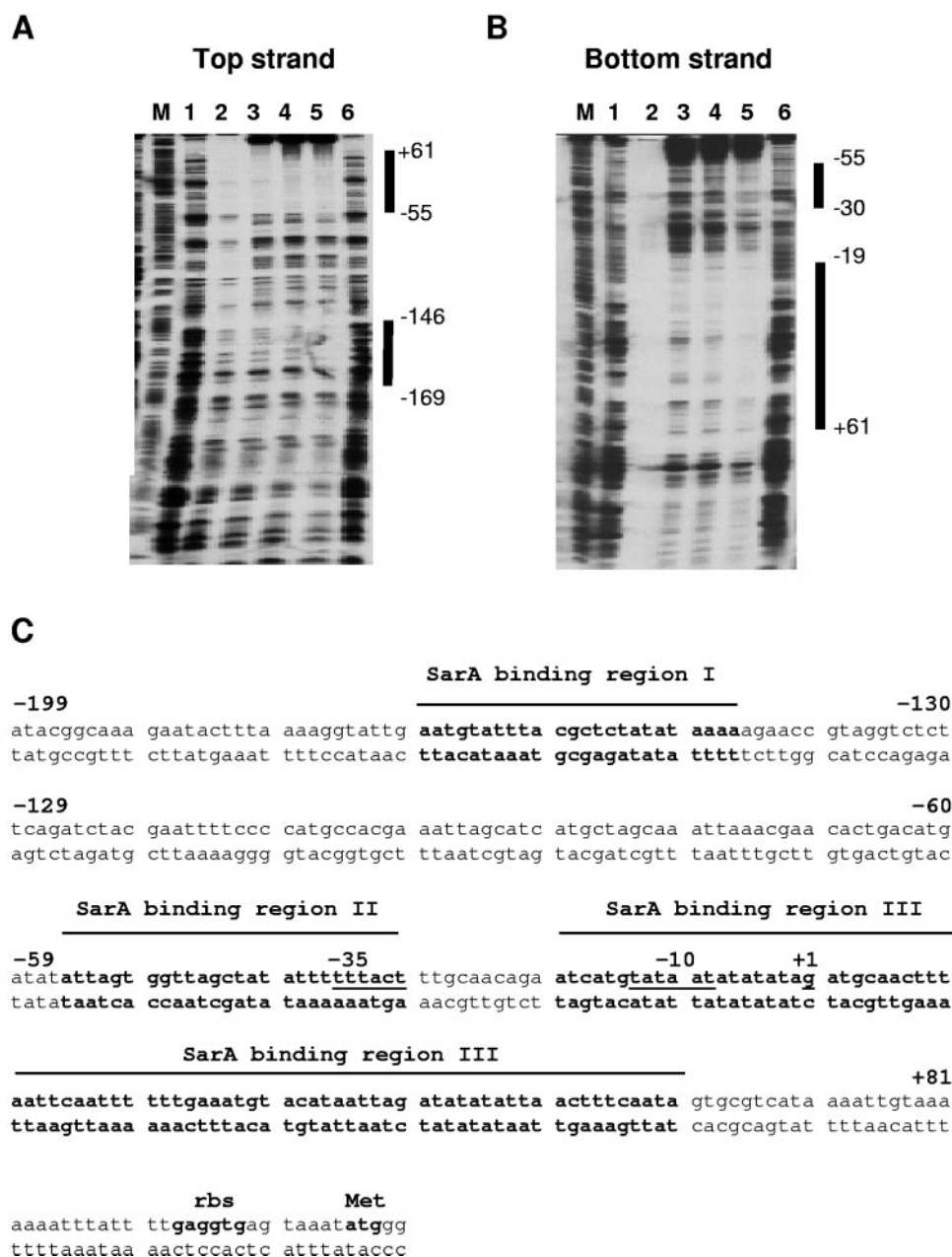


FIG. 6. DNase I footprinting analysis of SarA binding to the *bap* promoter region. (A) Footprint analysis of SarA binding to the top strand of the *bap* promoter region. A 295-bp *bap* promoter fragment was end labeled (top strand) with [ $\gamma$ - $^{32}$ P]ATP. The protein lanes (lanes 2 to 5) (0.25, 0.5, 1, and 2  $\mu$ g of SarA) as well as no-protein controls (lanes 1 and 6) are shown. The A to G ladder is also labeled (M). (B) Footprinting analysis of the bottom strand of the *bap* promoter region. The lanes contain 0.5, 1, and 2  $\mu$ g of SarA (lanes 3 to 5) or no-protein control (lanes 1 and 6). (C) Nucleotide sequence of the *bap* gene is shown and marked with the putative promoter region and start site as derived from the 5'-RACE data. The putative protected regions for SarA on the promoter are marked.

the main positive regulator of Bap expression. Interestingly, we and other have identified SarA as essential for the *ica*-dependent biofilm formation process, both in *S. aureus* (4, 41) and in *S. epidermidis* (39). Interestingly, Beenken et al. also disclosed that the unidentified factor responsible for the biofilm-positive phenotype of strain UAMS-1 was regulated by SarA (5). Taken together, these results strongly suggest that SarA is an essential regulator controlling biofilm formation in *S. aureus* by several mechanisms.

Several recent studies have shown discrepancies in the regulatory roles of *sarA* and  $\sigma^B$ . While we and others described *sarA* and not  $\sigma^B$  as a positive regulator of biofilm formation (4, 41), Pratten et al. established that *sarA* mutation increased adherence to glass (33). Contrary to our data (41), Rachid et al. suggested that  $\sigma^B$  was required for biofilm formation (34). It is conceivable that use of genetically unrelated strains may account for these discrepancies, since some studies have recently demonstrated the existence of strain-dependent differ-

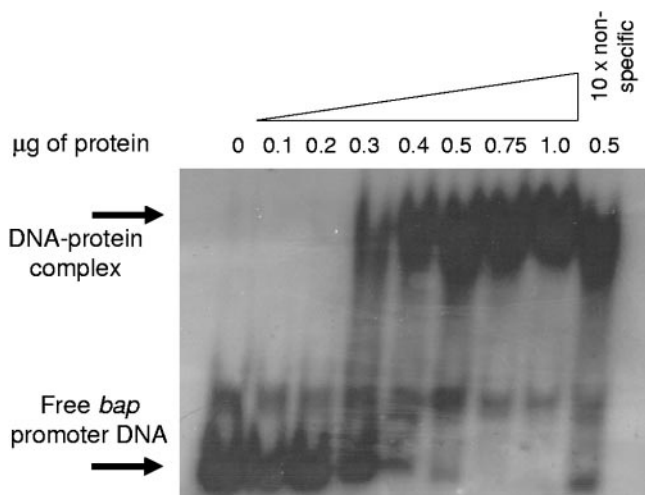


FIG. 7. Binding of SarA to *bap* promoter. Mobility of the DNA band in the presence of increasing amounts of SarA protein is indicated on the top. In competition assays, 10-fold excesses of nonspecific unlabeled DNA fragments were added with 500 ng of SarA.

ences in the regulatory roles of *sarA* and *agr* in *S. aureus* (6). Another plausible explanation may be that spontaneous mutations in global regulator genes may occur in some isolates of *S. aureus* as has been found in *S. epidermidis* (42, 43, and our unpublished results). For these reasons, to determine whether the phenotypes of strains V329 and c104 and the impact of *sarA* are representative and independent of the strain used, we expressed Bap in different genetic backgrounds, including strains Newman and SH1000. In strains Newman and SH1000, global regulators, including *agr*, *sar*, and  $\sigma^B$ , are fully functional. Interestingly, in all cases, a *sarA* mutation resulted in reduced capacity to express Bap and form biofilm. This finding confirmed the important role of SarA in controlling Bap expression.

Extracellular proteases expressed by *S. aureus* have been shown to degrade proteins on the surface of the bacteria (23, 28). Because protease production is upregulated in *sarA* mutants (8), we assessed if the decreased expression of Bap on the surface of the bacteria might be attributable to proteolytic cleavage of the protein. As the *sar-aur* and *sar-ssp* double mutants had defects in biofilm formation similar to the single *sar* mutant, we conclude that SarA-regulated proteases have little effect on biofilm formation in *sarA* mutants. Additionally, differential expression of cell-wall-associated Bap was mainly detectable during the exponential phase, a part of the growth cycle in which the secretion of proteases is not dominant (unpublished observation). However, because there are multiple proteases in *S. aureus*, we cannot rule out the possibility that enhanced proteolytic activity in the *sarA* mutant may contribute to the overall decrease in Bap-mediated biofilm formation.

Several studies have demonstrated that SarA modulates the expression of a number of *S. aureus* virulence factors, including *clfB*, *agr*, *fnbA*, *fnbB*, *spa*, *hla*, and *sspA* (10). In many cases, this regulation occurs via a direct interaction between SarA and promoter elements of the target genes. In gel shift studies we found that purified SarA protein formed complexes with the *bap* promoter with fairly high affinity. The specificity of the

SarA protein was determined by DNase I footprinting. Three SarA binding regions (boxes I, II, and III) were found within the 295-bp *bap* promoter sequence. Interestingly, each of these three binding regions resembles the SarA consensus binding motif or the SarA box (12). These findings are consistent with the notion that SarA may activate *bap* via direct binding into the *bap* promoter region.

In a previous study, we demonstrated that expression of the Bap protein blocked the activity of two MSCRAMM proteins of the *S. aureus* and reduced colonization capacity of Bap-expressing strains (16). In that study, the blocking capacity of the Bap protein was observed using stationary-phase cells. However, here we showed that Bap is expressed during the exponential phase of growth, as are the fibronectin-binding proteins (37), the collagen-binding protein (20), and the clumping factor B (29). Bap expressed during this phase of growth will block MSCRAMM activity, suggesting additional roles for this protein in staphylococcal pathogenesis to compensate the activity of the blocked proteins. Interestingly, an extracellular module, HYR, involved in cellular adhesion is present in the C repeats of Bap (20).

Bacteria seem to initiate biofilm development in response to a variety of environmental signals, such as nutrient and oxygen availability, osmolarity, temperature, or pH. Interestingly, in addition to the genetic control here reported, we found that addition of millimolar amounts of calcium to the growth media inhibited intercellular adhesion and biofilm formation by Bap-positive strain V329 (1). Our results also demonstrated that the  $\text{Ca}^{2+}$  inhibition of the Bap-mediated bacterial multicellular behavior was not due to repression of Bap expression. Instead, our results were consistent with the hypothesis that calcium causes a conformational change in Bap that affects its ability to form biofilms (1). The fact that calcium inhibition of Bap-dependent biofilm formation takes place in vitro at concentrations similar to those found in milk serum supports the possibility that this inhibition could be important to the pathogenesis of the bacteria.

To the recently characterized roles of the SarA protein in the regulation of biofilm formation (4, 5, 41), we have now added a role for *sarA* in the upregulation of Bap expression and biofilm development. The regulation of these processes by SarA suggests that SarA may be a promising target to control biofilm development and the infective process of *S. aureus*.

#### ACKNOWLEDGMENTS

This work was supported by grant BIO2002-04542-C02-01 from the Comisión Interministerial de Ciencia y Tecnología (C.I.C.Y.T.) as well as grants from the Cardenal Herrera-CEU University, from the Conselleria de Agricultura, Pesca i Alimentació (C.A.P.A.), and from the Generalitat Valenciana (CTIDIA/2002/62, CTESPP/2003/027 and AE04-8) to J.R.P., as well as grants from the NIH (AI37142 and AI47441) to A.L.C. Fellowship support for M. Pilar Trottonda from the Cardenal Herrera-CEU University is gratefully acknowledged.

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