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Bap, a *Staphylococcus aureus* Surface Protein Involved in Biofilm Formation

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Identification of new genes involved in biofilm formation is needed to understand the molecular basis of strain variation and the pathogenic mechanisms implicated in chronic staphylococcal infections. A biofilm-producing *Staphylococcus aureus* isolate was used to generate biofilm-negative transposon (Tn917) insertion mutants. Two mutants were found with a significant decrease in attachment to inert surfaces (early adherence), intercellular adhesion, and biofilm formation. The transposon was inserted at the same locus in both mutants. This locus (*bap* [for biofilm associated protein]) encodes a novel cell wall associated protein of 2,276 amino acids (Bap), which shows global organizational similarities to surface proteins of gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and gram-positive (*Enteroccocus faecalis*) microorganisms. Bap's core region represents 52% of the protein and consists of 13 successive nearly identical repeats, each containing 86 amino acids. *bap* was present in a small fraction of bovine mastitis isolates (5% of the 350 *S. aureus* isolates tested), but it was absent from the 75 clinical human *S. aureus* isolates analyzed. All staphylococcal isolates harboring *bap* were highly adherent and strong biofilm producers. In a mouse infection model *bap* was involved in pathogenesis, causing a persistent infection.

Staphylococcus aureus is one of the most important pathogens in humans and animals. The pathogenesis of a particular *S. aureus* strain is attributed to the combined effect of extracellular factors and toxins, together with the invasive properties of the strain such as adherence, biofilm formation, and resistance to phagocytosis. Despite general agreement that biofilms are the basis for persistent or chronic bacterial infections (8), the understanding of the molecular mechanisms implicated in the biofilm formation process is still growing (39). Two steps appear to be involved in this process: (i) attachment of the bacterial cells to a surface (early adherence) and (ii) growth-dependent accumulation of bacteria in multilayered cell clusters (intercellular adhesion) (18). Different proteins (24, 37), including those of the family of microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) (13), are involved in *S. aureus* adhesion. Specifically, MSCRAMMs may mediate *S. aureus* attachment to different cell types (11, 44) and abiotic surfaces once the adhesive host plasma constituents have covered the target surface (10, 29). However, the possible role of known MSCRAMMs or other molecules on the binding of *S. aureus* to inert surfaces in the absence of host constituents has not been thoroughly studied so far.

The *icaABCD* cluster, an operon present in *Staphylococcus epidermidis* and *S. aureus* (9, 20), participates in the intercellular adhesion step of biofilm formation by encoding proteins involved in the synthesis of the biofilm matrix polysaccharide poly-*N*-succinyl β-1-6 glucosamine (PIA-PNSG). The implication of *ica* in staphylococcal pathogenesis has been demonstrated in various animal models (30, 40). A relationship has been shown in vivo between the expression of *ica* in clinical *S. epidermidis* strains and infection (15). In addition, immunizations with PIA-PNSG efficiently protect against *S. aureus* infection (30).

Considerable effort has been made by different groups to associate *S. aureus* biofilm formation with different mechanisms of virulence and pathogenesis. In this context and using highly adherent strains, we have observed that *S. aureus* in vitro adherence and biofilm formation on inert or mammalian cell surfaces is associated with (i) exopolysaccharide production (6, 23); (ii) rough colony morphology phenotype in Congo red agar (CRA) (6); (iii) higher resistance to phagocytosis (32); (iv) lower susceptibility to antibiotics when forming biofilms (2); (v) higher capacity to attach to different surfaces and biomaterials used in orthopedic surgery, causing osteomyelitis (16); and (vi) higher capacity to colonize the ovine mammary gland, causing mastitis (6). In addition, active immunizations with exopolysaccharides extracted from a highly adherent *S. aureus* isolate have been shown to trigger protective immunity against mastitis (3). However, the genetic mechanisms underlying these observations have not been determined.

Transposon mutagenesis has been used to determine the genetics of biofilm formation in different bacterial species, including *S. epidermidis* (18, 33), *Streptococcus gordonii* (26), *Escherichia coli* (38), *Pseudomonas fluorescens* (36), and *Pseudomonas aeruginosa* (35), using polystyrene microtiter plates as substrate. With this methodological approach, we were able to identify in this study a new protein which is involved in *S.*

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aureus attachment to abiotic surfaces and biofilm formation in vitro and enhances infection in vivo.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. In order to find a strong biofilm forming strain, the ability of 350 *S. aureus* mastitis isolates to attach and form a biofilm on polystyrene microtiter plates was tested as previously described (9). Accordingly, a bovine subclinical mastitis isolate, *S. aureus* V329, was selected because of its strong biofilm production phenotype and antibiotic susceptibility profile, which facilitates genetic manipulations. This isolate was used to generate a genomic library and to obtain biofilm-negative transposon insertion mutants. *S. aureus* SA113, a restriction-negative and biofilm producer strain, and the biofilm-defective *S. aureus* SA113Δ*ica*, which contains a tetracycline resistance cassette that replaces the *ica* genes, were kindly provided by F. Götz (9). *S. aureus* RN4220, a restriction-negative strain, unable to form a biofilm, was kindly provided by J. C. Lee. Seventy-five human *S. aureus* strains and 50 coagulase-negative *Staphylococcus* strains from animals with bovine mastitis were analyzed for the presence of *bap*.

Staphylococcal strains were cultured in Trypticase soy agar (TSA) and in trypticase soy broth (TSB) supplemented with glucose (0.25%, wt/vol) when indicated. B2 broth (42) was used for biofilm formation on a glass surface. *E. coli* strains DH5α and BL21(DE3) were grown in Luria-Bertani medium. Media were supplemented when appropriate with erythromycin (20 μg/ml), ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml for plasmid pBT2 [7] and 20 μg/ml for plasmid pID408 [31]). Plasmid pID408 contains the transposon Tn917, which includes the pBR322 *amp or rop* region that allows replication and selection of the plasmid in *E. coli*, and the temperature-sensitive replicon pE194ts and the Cm^r gene of pTV32ts that allows replication and selection in *S. aureus* at 32°C. Plasmid pET-15b (Novagen) was used for protein expression in *E. coli*. The lambda vector EMBL-4 (Promega) was used to obtain a genomic library.

DNA manipulations. Routine DNA manipulations were performed using standard procedures (41). Plasmid DNA was isolated from *S. aureus* strains using a Qiagen plasmid miniprep kit according to the manufacturer's protocol, except that the bacterial cells were lysed by lysostaphin (12.5 μg/ml; Sigma) at 37°C for 2 h before plasmid purification. Plasmids were transformed into staphylococci by electroporation, using a previously described procedure (25) with the following modifications: 10% glycerol was replaced by 0.5 M sucrose, and staphylococcal transformations were enhanced by inducing chloramphenicol acetyl transferase translation with subinhibitory concentrations of chloramphenicol (0.2 μg/ml) after electroporation.

For Southern hybridization, chromosomal DNA was purified as previously described (28), digested with *Eco*RI, and analyzed by agarose gel electrophoresis. Gels were blotted onto nylon membranes (Hybond-N 0.45-mm-pore-size filters; Amersham Life Science) using standard methods (41). A 971-bp PCR fragment (oligonucleotides bap-6m, 5′-CCCTATATCGAAGGTGTAGAATTG CAC-3′ [1807], and bap-7c, 5′-GCTGTTGAAGTTAATACTGTACCTGC-3′ [2777]) of the *bap* region was used as a DNA probe (numbers in parentheses correspond to the position in the gene of the first nucleotide contained in the PCR fragment). Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labeling and chemiluminescence detection kit (Roche).

All the enzymes for DNA manipulation were supplied by MBI Fermentas, Roche, and Amersham Pharmacia Biotech; assays were performed as recommended by the manufacturer. Oligonucleotide primers were purchased from Life Technologies.

Transposon mutagenesis. *S. aureus* strain V329 harboring pID408 (V329:pID408) was grown overnight in TSA-chloramphenicol at 30°C. A single colony of V329:pID408 was inoculated in 1 ml of TSB-chloramphenicol (5 μg/ml) and incubated for 1 h at 30°C. Subsequently, 100 μl of this culture were spread on TSA-erythromycin plates and incubated for 18 h at 44°C. Transposon insertion mutants were subcultured on TSA-erythromycin plates. To exclude the possibility of contamination, mutants were compared with the parental strain by coagulase DNA typing (21).

Adherence studies. (i) Early adherence to an inert surface. Early adherence to a polystyrene surface was determined as previously described (14), with the following modifications. *S. aureus* strains were grown overnight in TSB supplemented with 0.25% glucose at 37°C. Overnight cultures were adjusted with TSB–0.25% glucose to an optical density at 578 nm (OD_{578}) of 0.1. Ten milliliters of each suspension was added to two polystyrene petri dishes and incubated for 1 h at 37°C. Petri dishes were washed at least five times with phosphate-buffered saline (PBS). Cells were fixed with Bouin solution and Gram stained. Adherent bacterial cells were observed by oil immersion microscopy and counted (results represent the means of four different microscopic fields). Each experiment was repeated three times.

(ii) Biofilm assay. A late adherence assay was carried out essentially as described elsewhere (18). Briefly, *S. aureus* strains were grown overnight in TSB at 37°C. The culture was diluted 1:40 in TSB–0.25% glucose, and 200 μl of this cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Iwaki). After 12 h, medium was replaced, and 12 h later, the wells were gently washed three times with 200 μl of sterile PBS, 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 and repeated five times.

Verification of the classification of strains as biofilm producers and nonproducers was carried out by different methods.

(a) Formation of cell aggregates in a cell suspension. Cells were grown overnight in 5 ml of TSB–0.25% glucose at 37°C and examined macroscopically and microscopically for the presence or absence of aggregates (intercellular adhesion).

(b) Macroscopic observation of biofilm on glass. Cells were grown in 50 ml of B2 at 37°C, using a glass container, without shaking, for 2 days, and the walls of the container were visually (macroscopically) examined for the presence or absence of a white biofilm layer.

(c) Colonization of other materials. The capacity to form a biofilm layer was verified using polyvinylchloride (PVC) plastic as a target surface and a phase-contrast microscope (magnification, ×1,000; Nikon Optiphot-2 microscope), as previously described (38).

(d) Colony morphology in CRA. Colony morphology was determined in CRA as previously described (6, 48), with rough colonies being indicative of biofilm formation (positive result) and smooth colonies being associated with a deficiency in biofilm formation.

PIA-PNSG detection. PIA-PNSG production in *S. aureus* was detected as described by Cramton et al. (9). Briefly, cells were grown overnight in TSB supplemented with 0.25% glucose, the optical density was determined, and the same number of cells (2 to 4 ml) from each culture was resuspended in 50 μl of 0.5 M EDTA (pH 8.0). Cells were then incubated for 5 min at 100°C and centrifuged to pellet the cells, and 40 μl of the supernatant was incubated with 10 μl of proteinase K (20 mg/ml; Sigma) for 30 min at 37°C. After addition of 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 10 μl was spotted on a nitrocellulose filter using a Bio-Dot Microfiltration apparatus (Bio-Rad), blocked overnight with 5% skim milk in PBS with 0.1% Tween 20, and incubated for 2 h with an anti-*S. aureus* PIA-PNSG antibody diluted 1:10,000 (30). Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:10,000, and the Amersham ECL (enhanced chemiluminescence) Western blotting system.

Gene identification. A previously described method (31) was used to clone the chromosomal DNA flanking the transposon insertion points in mutants with attenuated biofilm production. Briefly, 2.5 μg of *S. aureus* chromosomal DNA from each mutant was restricted with *Eco*RI, resuspended in 200 μl of ligation buffer (Promega), and self-ligated for 12 h at 16°C. The ligated products were transformed into *E. coli* DH5α, plated onto Luria-Bertani agar containing ampicillin, and incubated at 37°C overnight. Plasmid DNA was extracted from a single ampicillin-resistant colony using a Qiagen plasmid miniprep kit. Chromosomal DNA sequences flanking the transposon were obtained using primer Tn*917*-3c (5′-AGAGAGATGTCACCGTCAAGT-3′), which corresponds to the inverted repeat region located 70 bp from the *erm*-proximal end of Tn*917*.

Construction of genomic library. The lambda vector EMBL-4 was used to construct a genomic library of *S. aureus* V329 according to the manufacturer's instructions (Promega). Chromosomal DNA of *S. aureus* V329 was digested with *Eco*RI and ligated into vector EMBL-4 restricted with *Eco*RI. A 200-bp PCR fragment of the *bap* flanking region was used as a DNA probe (oligonucleotides 556-1m, 5′-CTGTCCATATTTGGACTGTG-3′, and 556-2c, 5′-CTTATAGAT GTGCGTAGTC-3′). Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labeling and chemiluminescence detection kit (Roche).

DNA sequencing and computer analysis. A genomic *Hin*dIII fragment including the *bap* gene was cloned in pBT2 plasmid (pBT2:Bap). The nucleotide sequence was determined by the dideoxy chain termination method, using an ABI 377 model automatic sequencer (PE Biosystems; Foster City, Calif.) at the DNA Sequencing Service of the IBMCP-UPV (Valencia, Spain). For C-repeat sequencing, a genomic DNA *Xba*I-*Eco*RI fragment containing this region was subcloned in pBT2. Nested deletions were generated (Erase-a-base system; Promega). Double DNA sequencing of this region was carried out using the primers pBT2-1c (5′-GGACGATATCCCGCAAGAGGCCCG-3′) and pBT2-2c (5′-GG

TGCCGAGGATGACGATGAGCGC-3′), corresponding to the flanking region of the plasmid pBT2 cloning site.

Homology searches were carried out using the BLAST 2.0 program (1) at the NCBI server. The cloned sequence was compared against those in the GenBank database and the publicly available *S. aureus* genomes (The Institute for Genomic Research, University of Oklahoma, and Sanger Centre).

Complementation studies. To prove that the biofilm-deficient phenotype of the mutants was due to the disruption of *bap*, mutants were complemented with plasmid pBT2 carrying a 9.2 kb *Hin*dIII fragment from the wild-type strain, including the *bap* gene under the control of its own promoter (pBT2:Bap). Plasmid pBT2:Bap was transformed by electroporation into *S. aureus* strains m556, RN4220, SA113, and SA113Δ*ica*. Stable expression of Bap was analyzed in total bacterial extracts by Coomassie staining of proteins run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

SDS-PAGE and Western blot analysis. Bacteria were grown overnight in TSB (supplemented with 10 μg of chloramphenicol/ml in the case of complemented strains) at 30°C. Following centrifugation of 1 ml of culture, cells were harvested, washed, and finally resuspended in 75 μl of PBS buffer containing lysostaphin (12.5 μg/ml; Sigma). After 2 h of incubation at 37°C, cells were resuspended in 1 volume of Laemmli buffer and boiled for 10 min. After centrifugation, 20 μl of the supernatant was used for SDS-PAGE (10% separation gel, 4.5% stacking gel) and proteins were stained with Coomassie brilliant blue R250 (0.25%; Sigma).

For Western blot analysis, protein extracts were prepared and analyzed by SDS-PAGE as described above and blotted onto Immobilon P membrane (Millipore). Anti-Bap serum was diluted 1:2,500 with Tris-buffered saline (TBS) (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) and immuno-absorbed with 5% skim-milk. Alkaline phosphatase-conjugated goat anti-rabbit Immunoglobulin G (Sigma) diluted 1:15,000 in TBS–5% skim milk was used and the subsequent chemiluminescence reaction (with CSPD [Roche]) was recorded.

Expression of the N-terminal region of Bap in *E. coli*. A 1,919-bp DNA sequence corresponding to nucleotides (nt) 1 through 1919 of the *bap* gene was amplified by PCR with primers bap-2m (5′-GGGGGG**CATATG**GGAAATAA ACAAGGTTTTTTACC-3′) and bap-3c (5′-GGGG**GGATCC**CCCAACCTCGTC AATGGTTAAGTCAGC-3′) (*Nde*I and *Bam*HI restriction sites are shown in boldface type). The amplified product was restricted with *Nde*I and *Bam*HI and cloned in frame downstream from the His tag sequence in the pET-15b vector. The nucleotide sequence of the cloned *bap* fragment was verified by sequence analysis. Purified plasmid DNA was used to transform the expression host BL21(DE3), and the fusion protein was produced as specified by the manufacturer (Novagen). After disrupting the cells by sonication, the recombinant protein was purified by immobilized metal affinity chromatography using a cobalt-based resin (Clontech).

Production of rabbit polyclonal antiserum. Polyclonal antiserum to purified recombinant protein was raised as previously described (43). For the initial dose (day 1), 100 μg of antigen in complete Freund's adjuvant was injected subcutaneously. Booster doses (50 μg) were administered intramuscularly on days 14 and 42 in incomplete adjuvant. Blood was collected from the marginal ear vein at 2-week intervals after booster administration, and antibody titer in serum was determined.

Experimental infection. A mouse foreign body infection model was used to determine the role of *bap* in the pathogenesis of *S. aureus*. A total of 53 adult male mice (Swiss-Albino, B&K Universal, Barcelona, Spain) were used. A 1-cm segment of intravenous catheter (22G1″; B. Braun) was aseptically implanted into the subcutaneous interscapular space. Each group of nine mice was inoculated with 1.5×10^5 CFU of either *S. aureus* V329 or the Bap-defective *S. aureus* strain m3591. Three animals were euthanatized by cervical dislocation on days 4, 7, or 10 postinfection. The catheter was aseptically removed, placed in a sterile microcentrifuge tube containing 1 ml of PBS, and vortexed at high speed for 3 min. The number of bacteria was determined by plate count. The experiment was repeated three times.

In each experiment, an extra group of animals was inoculated with vehicle (PBS) and served as a negative control. In addition, to exclude the possibility of contamination, bacteria recovered at the end of the experimental period were compared with the parental strains by coagulase DNA typing (21) and with regard to growth in TSA-erythromycin, CRA colony morphology phenotype, and biofilm formation capacity (see above).

Statistical analysis. A two-tailed Student's *t* test was used to determine the differences between groups in biofilm formation. A nonparametric test (Mann-Whitney U test) was used to assess significant differences in bacterial recovery within groups in primary adherence and experimental infection. For analysis of the cure ratio, a two-by-two contingency table was produced and Fischer's exact test was applied. Differences were considered statistically significant when P was <0.05 in all cases.

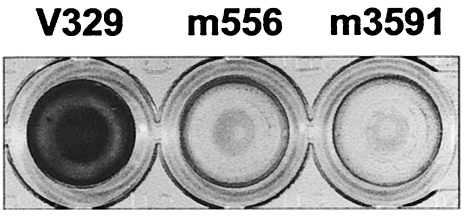


FIG. 1. Biofilm formation phenotype. Differences between the wild-type strain V329 and mutants m556 and m3591 in the capacity to form a 24-h biofilm on polystyrene microtiter plates after staining with safranin. The ELISA plate colors correspond to OD_{490}s of 1.3, 0.17, and 0.18, respectively.

Nucleotide sequence accession number. The DNA sequence reported in this article has been deposited in the GenBank nucleotide sequence database under accession number AF288402.

RESULTS

Production and characterization of Tn*917* mutants. Upon transfer of plasmid pID408 into the adherent *S. aureus* strain V329, a collection of approximately 4,000 random Em^r Cm^s transposon insertions was screened for their ability to form a biofilm. Two mutants, designated m556 and m3591, had lost the ability to form a biofilm (Fig. 1) and exhibited a growth rate indistinguishable from that of the wild type. Southern hybridization analysis of *Eco*RI-digested chromosomal DNA using a Tn*917*-specific probe revealed that each mutant had a single transposon insertion (data not shown).

Sequencing of the DNA flanking the transposon insertion sites in both mutants revealed that the transposon insertions disrupted the same gene, but at different positions. When the DNA sequences were compared with those in the GenBank and the *S. aureus* databases using the BLAST program, no significant similarity with any sequence was found.

The putative gene involved in biofilm formation was cloned from a lamba genomic library as a 16-kb fragment detected by hybridization to the region flanking the transposon insertion site. The nucleotide sequence of this 16-kb fragment revealed that the transposon disrupted an unusually large gene, designated *bap* (coding for a biofilm-associated protein, Bap), consisting of 6,831 nt and harboring a potential ribosome-binding site (TGAGG) located 9 nt upstream of the ATG initiation codon. The presence of a putative promoter and transcription terminator sequence upstream and downstream of *bap* suggests that this gene is not part of an operon. Hence, polar effects of the transposon insertion are unlikely. The deduced amino acid sequence of Bap consists of 2,276 amino acids (aa), with a theoretical molecular mass of ≈239 kDa.

Bap expression allows and enhances biofilm formation. A double band that migrated at a position corresponding to 230 and 240 kDa was reproducibly detected by SDS-PAGE of the total protein extract from the wild-type bacteria but not from the m556 mutant (Fig. 2A). Bands of similar mobility were also detected in *S. aureus* strains which were complemented with the *bap* gene in pBT2:Bap. In addition, a band of the expected size was recognized by polyclonal antibodies raised against the first 640 aa of Bap (Fig. 2B), strongly suggesting that the band

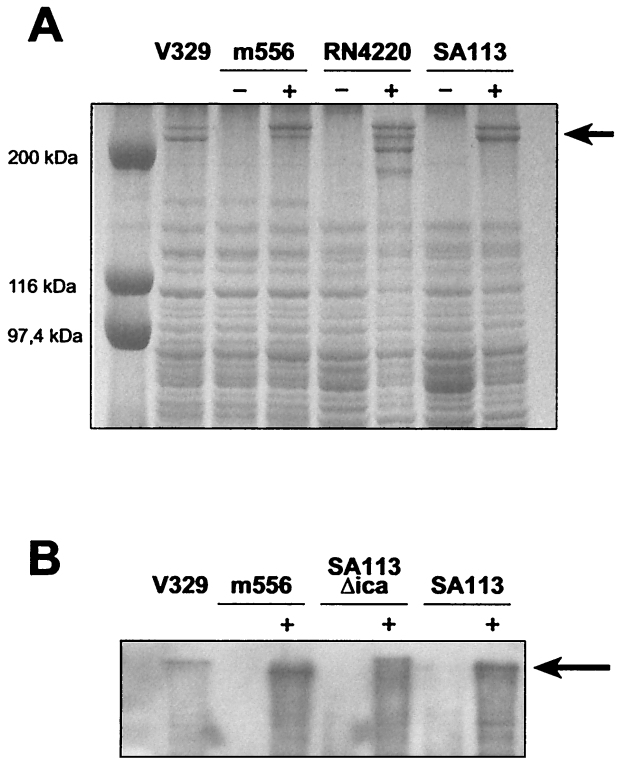


FIG. 2. (A) SDS-PAGE of protein extracts of wild-type strain V329, m556, RN4220, SA113, and the corresponding complemented strains: m556 pBT2:Bap (m556, lane +), RN4220 pBT2:Bap (RN4220, lane +), and SA113 pBT2:Bap (SA113, lane +). Note that a double protein band of 230 and 240 kDa is present in the wild-type strain as well as in all the strains harboring plasmid pBT2:Bap. (B) Study of the presence of Bap by Western blotting. An approximately 240-kDa band is recognized by polyclonal antibodies against the first 640 aa of Bap only in the wild-type strain V329 and complemented strains: m556 pBT2:Bap (m556, lane +), SA113Δ*ica* pBT2:Bap (SA113Δica, lane +), and SA113 pBT2:Bap (SA113, lane +).

present in the wild type and complemented strains is the product of the *bap* gene.

Analysis of the capacity to form a biofilm (on a polystyrene surface after 24 h) demonstrated that all strains expressing Bap, including *S. aureus* SA113, a strain whose biofilm formation has been related to the *ica* product (PIA-PNSG), showed an enhanced capacity to form a biofilm, implicating Bap in this process (Fig. 3). Significant differences in adherence were detected between the parental strain and its isogenic mutant, as well as between noncomplemented and complemented strains ($P < 0.01$). It is important to note that the capacity for biofilm formation was not completely restored in the complemented m556 mutant, although the expression level of the protein was similar to that in the wild type.

Bap is involved in primary adherence and intercellular adhesion. Primary adherence of *S. aureus* strains V329, m556, SA113, and SA113Δ*ica* is illustrated in Fig. 4. *S. aureus* V329 adhered to polystyrene much more efficiently than the isogenic *bap* mutant m556 and strains SA113 and SA113Δ*ica*. In addition, the presence of cell-to-cell clusters was observed in V329 and SA113 cultures (incubated overnight in TSB–0.25% glucose) by phase-contrast microscopy (data not shown), but not in the mutant m556 and SA113Δ*ica* cultures. Accumulation of cell aggregates at the bottom of the tube was macroscopically observed only in the case of wild type strains. These results strongly suggest that Bap is not only involved in intercellular adhesion and accumulation in multilayered cell clusters, as the product of the *ica* operon does, but also in primary attachment to an abiotic surface.

Relationship between Bap and PIA-PNSG. To determine Bap–PIA-PNSG interaction we tested PIA-PNSG production in Bap^+ and Bap^- strains. The strain V329 showed a low level of in vitro PIA-PNSG production. Similar results were obtained whith other natural Bap^+ strains (data not shown). Inactivation of the *bap* gene in the mutant strain m556 reduced the levels of PIA-PNSG (Fig. 5). Complementation of the PIA-PNSG-producing strain SA113 with the *bap* gene strongly increased PIA-PNSG accumulation (Fig. 5). As expected, PIA-PNSG was no longer produced in the *ica* knockout strain SA113Δ*ica* (Fig. 5).

Further phenotypic characterization of biofilm-defective mutants. Consistent with the results on polystyrene microtiter plates, the wild-type strain (V329) grown on CRA exhibited a rough colony morphology typically associated with biofilm producers, whereas mutants m556 and m3591 produced a smooth colony morphology commonly found in non-biofilm-producing strains (Fig. 6A). Phase-contrast microscopic observation of late adherence to PVC plastic discs showed that the wild-type strain produced multiple layers of cells almost completely covering the PVC surface (Fig. 6B). In contrast, very few cells of the biofilm-defective mutant were attached to PVC plastic. Macroscopic examination of biofilms in a glass container revealed that upon 2 days of culture, the wild-type strain formed an obvious biofilm on the glass surface, but the mutants did not (Fig. 6C).

Structural features of Bap protein. Analysis of the Bap primary amino acid sequence revealed the presence of a typical gram-positive amino-terminal signal sequence for extracellular secretion (first 44 aa) and a putative carboxy-terminal segment containing an LPXTG motif and a hydrophobic membrane-spanning domain followed by a series of positively charged residues typical of cell-wall-anchored surface proteins of gram-positive bacteria (34) (Fig. 7A). Following the putative signal peptide, the N-terminal region of Bap can be divided into two regions. Region A (aa 45 to 360) contains two short repeats of 32 aa (repeats A_1 and A_2) separated by 26 aa (Fig. 7B). BlastP searches of this region revealed no significant similarity scores among GenBank sequences. Region B, the remaining part of the N-terminal domain (aa 361 to 818), exhibited a significant similarity with an *Enterococcus faecalis* surface protein (Esp) (43), which is mostly found in infection-derived isolates.

The central region of Bap (aa 819 to 2147) begins with a spacer region (aa 819 to 947) followed by 13 nearly identical 258-nt tandem repeat units encoding reiterations of an 86-aa sequence (C repeats, aa 948 to 2139) (Fig. 7A). The C-repeat region begins with a partial C-repeat sequence corresponding to the last 37 aa of a C repeat and ends with a partial C-repeat sequence corresponding to the 37 first aa of a C repeat (Fig. 7B). The C-repeat region accounts for 52% of the Bap protein, and each repeat shows high sequence identity. BlastN searches of the 258 nt encoding a C repeat reveals a strong similarity with a partial GenBank sequence (*Pst*I *S. simulans*, G. Thumm

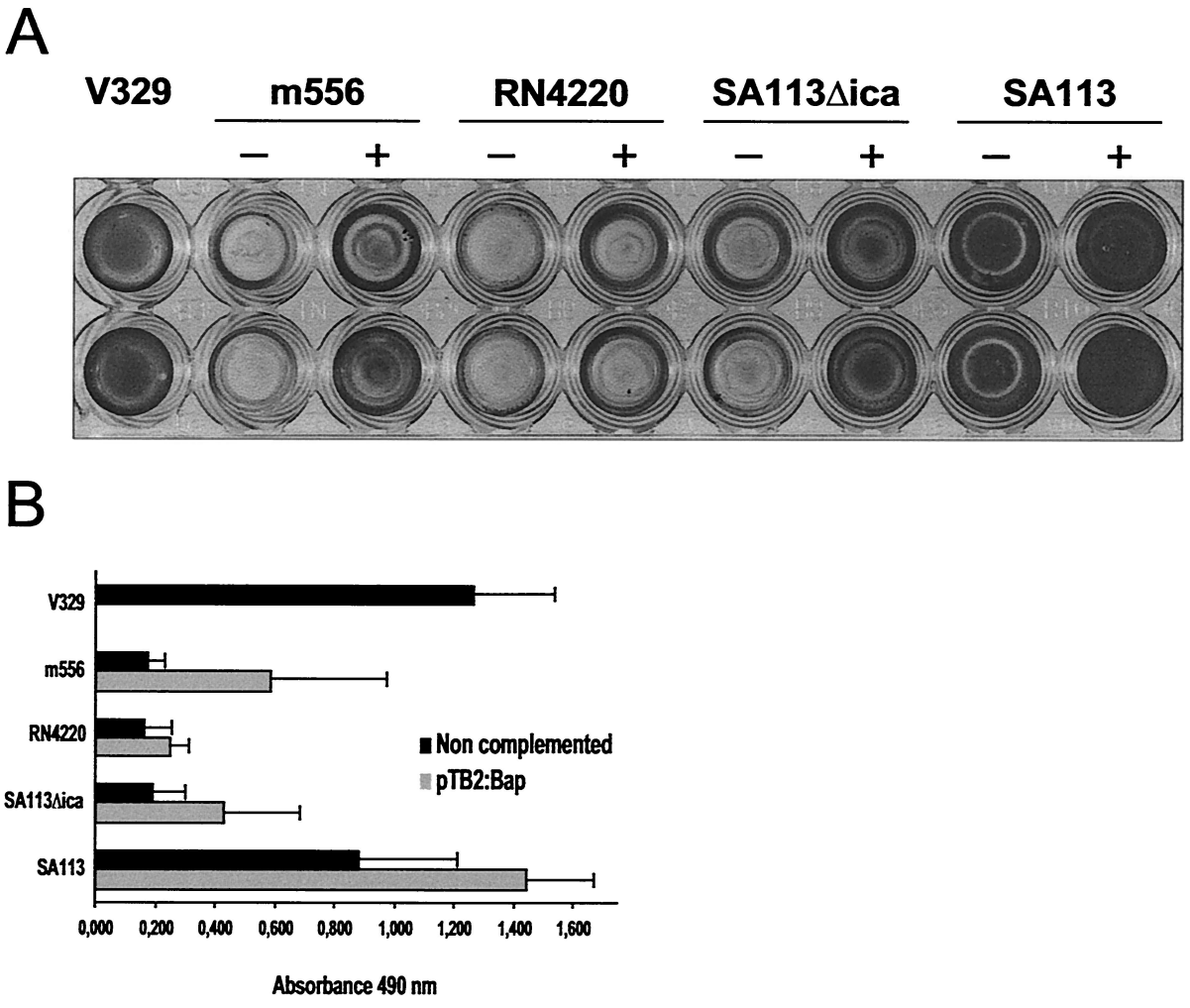


FIG. 3. pBT2:Bap complementation studies involving biofilm formation in biofilm-defective mutant m556, non-biofilm-forming strain RN4220, and biofilm-forming strain SA113 and its *ica*-defective mutant SA113Δ*ica*. Biofilm formation capacity differences correspond to 24-h biofilm formed on polystyrene microtiter plates after staining with 0.1% safranin. The ELISA plates and mean OD_{490}s obtained are shown. (A) ELISA wells corresponding to wild type V329, m556 (−) and m556 pBT2:Bap (+), RN4220 (−) and RN4220 pBT2:Bap (+), SA113Δ*ica* (−) and SA113Δ*ica* pBT2:Bap (+), and SA113 (−) and SA113 pBT2:Bap (+). (B) Mean optical density. Bars represent the mean values, and error bars represent the standard errors of the means ($n = 5$). Significant differences in adherence ($P < 0.01$) were noted between complemented and noncomplemented strains, as well as between the wild type and isogenic mutants.

and F. Götz, accession number U66881), while BlastP searches of the C repeats exhibited high similarity with extracellular proteins from different species, including *P. aeruginosa* (E value: e-120; accession number AAG05263), *Pseudomonas putida* (E value: 3e-94; accession number AF182515), *Synechocystis* sp. (E value: 6e-94; accession number D63999), *Salmonella enterica* serovar Typhi (E value: 1e-82; accession number AF139831), and *Enterococcus faecalis* (E value: 5e-81; accession number AF034779).

The carboxy-terminal region of Bap comprises the D region and the LPXTG motif (Fig. 7A). The D region (aa 2148 to 2208) consists of three short repeats of 18 aa followed by an incomplete repeat comprising the first 7 aa of the D repeats (Fig. 7B). LPXTG is a consensus cell wall anchor motif found in most wall-associated surface proteins of gram-positive bacteria (34). BlastP searches of the D region revealed no significant similarity scores among GenBank sequences.

When the Bap protein was compared with products of *S. aureus* genomes using the BLAST program, no significant similarity with any sequence was found.

Distribution of the *bap* gene among staphylococcal species. PCR amplification and Southern blot analysis using specific probes for the *bap* gene revealed that *bap* is present in only 5% of the 350 *S. aureus* bovine mastitis isolates tested. The presence of *bap* could not be detected in any of the 75 human *S. aureus* isolates studied. All the strains harboring *bap* were strong biofilm producers. Sequences that hybridized with the *bap* probe were present in 10% of the 50 coagulase-negative *Staphylococcus* isolates tested from animals with bovine mastitis (data not shown).

Experimental infection. Bap contributed to the pathogenesis of *S. aureus* in the murine catheter-induced infection model (Fig. 8). Although at day 4 postinoculation differences between wild-type and mutant strains in the number of recovered bac-

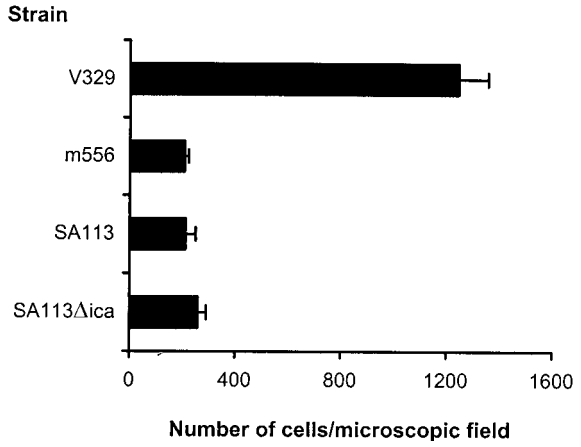


FIG. 4. Primary attachment assay. Significant ($P < 0.01$) differences were detected between wild-type strain V329 and isogenic mutant m556. No differences were found between *S. aureus* SA113 and isogenic mutant SA113Δ*ica*. Bars represent the mean values, and error bars represent the standard errors of the means ($n = 3$).

teria per catheter were non significant, at day 7 postinoculation, the number of recovered bacteria (CFU) was 1.2×10^6 and 2.8×10^5 for the wild-type and mutant strains, respectively ($P > 0.05$). This difference increased by day 10, when the values were 1.7×10^6 and 3×10^4 CFU, respectively ($P < 0.05$).

The analysis of the cure ratio (number of animals infected/number of animals inoculated) (Table 1) showed that animals infected with the wild-type strain developed a persistent infection (six of nine animals infected at day 10). However, animals inoculated with the mutant strain were more capable of eliminating the infection (two of nine animals infected at day 10). These results indicate that Bap is an important factor determining the persistence of infection.

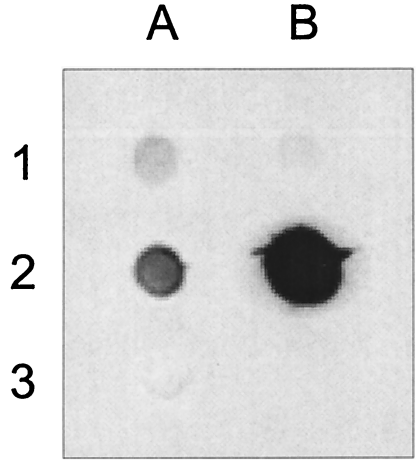


FIG. 5. Dot blot analysis of *S. aureus* PIA-PNSG acummulation induced by Bap expression. *S. aureus* V329 (blot A1) and m556 (blot B1) produced low levels of PIA-PNSG. Complementation of *S. aureus* SA113 with pBT2-Bap plasmid strongly increased the levels of PIA-PNSG (blot B2) with respect to the wild-type strain (blot A2). PIA-PNSG was not detected in *S. aureus* SA113Δ*ica* (blot A3).

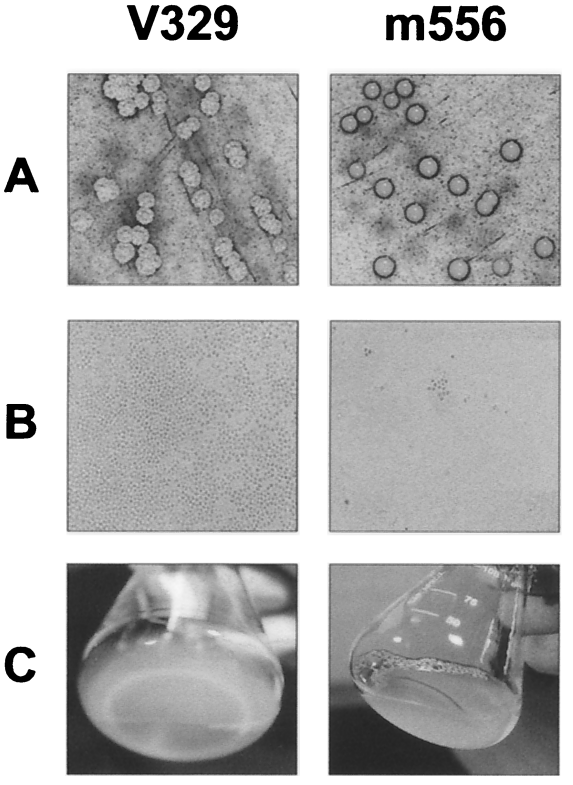


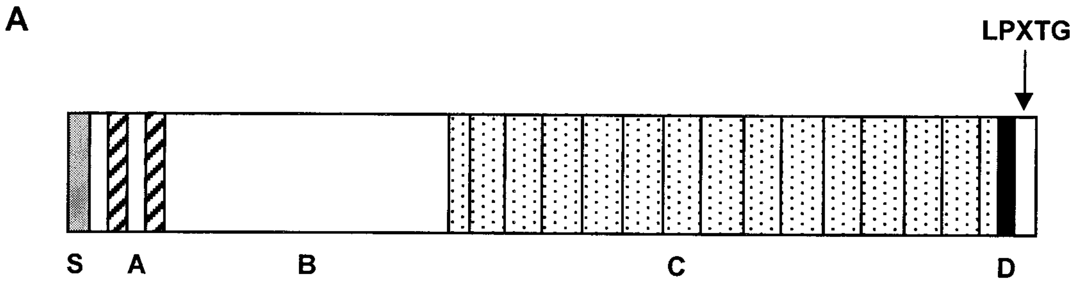
FIG. 6. Phenotypic differences between the wild-type strain V329 and the defective mutant m556. (A) CRA colony morphology; (B) capacity to form a 24-h biofilm on PVC plastic, as observed by phase-contrast microscopy (magnification, ×1,000); (C) capacity to form a 48-h biofilm on the surface of a glass container (visual observation).

DISCUSSION

In *S. epidermidis*, several surface proteins involved in biofilm formation have been described, three of which (SSP1, SSP2, and the AtlE autolysin [19, 45]) contribute to the primary attachment, and the other, a 140-kDa protein mediating intercellular adhesion, has been proposed as essential for accumulation of sessile bacteria on glass or polystyrene surfaces (22). The absence of homology among these proteins strongly suggests that staphylococci may use different approaches to form a biofilm. To our knowledge, data presented in this report describe the first *S. aureus* surface protein (Bap) directly involved in biofilm formation on abiotic surfaces in the absence of host plasma constituents.

To identify Bap, we have used the standard biofilm assay on microtiter plates for the screening of transposon mutants unable to adhere to the polystyrene surface. Surprisingly, in contrast to similar studies with other microorganisms like *P. aeruginosa* (35), *E. coli* (38), and *Vibrio cholerae* (47), where several genes were found to be involved in the biofilm formation process, this assay allowed identification of only two mutants in which the transposon affected the same 6,831-bp open reading frame designated *bap*.

The *bap* gene displayed little sequence similarity with known genes. However, the 2,276-aa *bap* product displayed an organizational similarity with an outer membrane protein of *P. putida* involved in adhesion to seeds (12) and with a surface



B

A repeats

```
AQDDDNIKED  SNTQEESTNT  SSQSSEVPQT  KK
AQDDDNIKED  SNTQEESTNT  SSQSSEVPQT  KK
```

C repeats

```
                                                                 D APTLDEVNTD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNGDLNG GEELQVTATD KDGNTSEPSS ANVTDTTASD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ASQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSEPSS ANVTDTTAPD APTVNDVTSD ATQVTGQAEP NSTVKLTFPD GTTATG
TADDQGNYTI DIPSNVDLNG GEELQVTATD KDGNTSE
```

D repeats

```
SDDNSDNGNN  SGAGDTSD
SDDNSGNGDN  SGAGDNSD
SDDNSDNGNN  SGAGDNSD
SDDNSDN
```

FIG. 7. (A) Structural analysis of Bap. S represents the signal sequence. The positions of the LPXTG motif and the A, B, C, and D domains are shown. Region A (aa 45 to 360) contains two short repeats of 32 aa (A_1 and A_2) separated by 26 aa. Region B (aa 361 to 818) represents the remaining part of the N-terminal domain. A spacer region (aa 819 to 947) followed by region C (aa 948 to 2139) consisting of 258-nt tandem repeats constitutes the central domain of Bap. The carboxy-terminal region of Bap consists of region D (aa 2148 to 2208), which contains three short repeats of 18 aa followed by an incomplete D repeat, and the LPXTG motif. (B) Alignment of the amino acid residues within repeat blocks A, C, and D of the Bap protein. Substitutions are shown in boldface type.

protein of *E. faecalis* (Esp) of unknown function (43), but whose presence is highly correlated with the ability of this bacteria to produce a biofilm on abiotic surfaces (our unpublished results). The most remarkable feature of Bap is the presence of an extensive repeat region, in which the repeats are identical even at the nucleotide level. Although the biological function of the repeats has not been established, it is tempting to speculate that this region could serve to project the amino-terminal part of the protein from the cell surface and to promote interaction with abiotic surfaces, host cell components, or other bacteria. Analogous long repeats have been described in the alpha C protein of group B streptococci (46), where addition or deletion of repeat units leads to the expression of variant proteins which allow bacteria to escape the host immune response (27). Similar pathogenic mechanisms might occur in different Bap$^+$ strains, in which a variable number of C repeats can be found in the structure of the *bap* gene.

Primary attachment, intercellular aggregation, and biofilm formation studies showed that Bap promotes primary attachment as well as the second step of biofilm formation, where intercellular adhesion plays an important role. In this context, since complementation of *S. aureus* SA113 with *bap* resulted in a significantly increased ability to form a biofilm and PIA-PNSG accumulation, it is tempting to speculate that PIA-PNSG and Bap may cooperatively affect cell-to-cell aggregation during the biofilm formation process. A possible explanation of the observation that biofilm formation capacity was not completely restored in the complemented m556 mutant is that the insertion of the transposon in mutant m556 resulted in the production of a truncated protein lacking the last 15 aa, and therefore the protein would be unable to anchor to the cell wall. The soluble truncated Bap protein may cover the abiotic surface and compete with the cell-associated Bap for the interaction with the abiotic surface.

CRA has been used to discriminate by colony morphology between biofilm and non-biofilm-forming strains of *S. aureus* (2, 6) and *S. epidermidis* (48), since they produce rough and

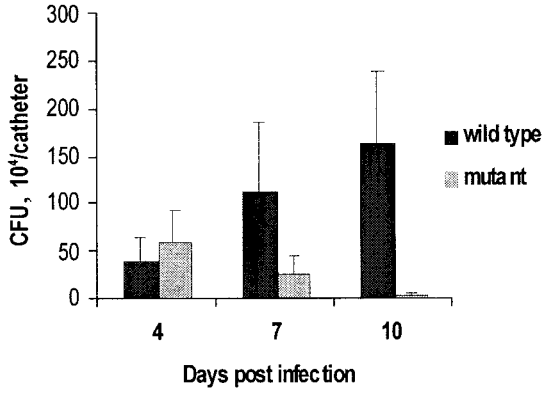


FIG. 8. Recovery of *S. aureus* V329 and its isogenic Bap mutant m3591 from implanted subcutaneous catheter in a mouse foreign body infection model. Bars represent the mean of CFU collected from catheters, and the error bars represent the standard errors of the means ($n = 9$). At day 10, differences between wild type and mutant were detected ($P < 0.05$). Bacteria were not detectable in control animals at the end of the experimental period.

smooth colonies, respectively. Congo red interacts with several proteins and proteinaceous fibrillar structures, such as curli fimbriae from *E. coli* (17) and the type III secretion machinery of *Shigella flexneri* (4). In the case of *bap*-harboring *S. aureus*, loss of the Bap protein resulted in transformation of the rough colony morphology (of the wild-type V329 strain) into the smooth colony morphology (of the m556 mutant). A similar phenotypic variation has been described in *S. epidermidis* strains deficient in the production of PIA-PNSG (the *ica* product) (48) and *S. aureus* strain SA113Δ*ica* (our unpublished results). When the expression of *bap* was restored in complemented strains, the rough phenotype appeared, suggesting the implication of Bap in this phenotypic variation. Why the deficiency in either the polysaccharide intercellular adhesin (PIA-PNSG) or a surface protein (Bap) results in the same phenotypic change is unknown.

Animal models have been useful to study the importance of different genes in the pathogenesis and virulence of *S. aureus* (30, 31). In this report, we used a mouse foreign body infection model to evaluate the role of Bap and the resulting biofilm formation process in the pathogenesis of *S. aureus*. Differences between the Bap-deficient mutant *S. aureus* m3591 and the parental strain in the capacity to colonize the catheter became more obvious at late stages of infection (by day 10), when the mutant strain showed a decreased persistence relative to the wild type. In the closely related species *S. epidermidis*, the expression of specific bacterial cell surface components appears to hinder the interaction of particular bacterial cell receptors with host proteins (5). In our model, the catheter may have become rapidly coated in vivo by host proteins after implantation and Bap might have hindered the interaction between bacterial receptors and the host proteins on the catheter. This may explain why a Bap-deficient mutant may be more prevalent than the wild-type strain at the initial stages of infection (up to day 4). Later on (days 7 to 10), biofilm formation would be strongly enhanced by Bap as infection proceeds, by promoting cell-to-cell interactions, bacterial accumulation, and persistence of infection.

TABLE 1. Analysis of cure ratio at various days postinoculation

Bacteria	Cure ratio[a]			
	Day 4	Day 7	Day 10	Total[b]
Wild type	6/9	6/8	6/9	18/26
Bap-deficient mutant	4/9	5/9	2/9	11/27

[a] Number of infected animals/number of inoculated animals.
[b] Differences between the wild-type and Bap-deficient mutant groups were significant, as determined by Fischer's exact test.

In conclusion, this study describes a novel protein of *S. aureus* involved in biofilm formation on abiotic surfaces. Attachment to abiotic surfaces might not be necessarily related to attachment to biotic surfaces. In fact, there are examples that support the idea that bacterial biofilm formation can proceed through divergent pathways, depending on whether or not bacteria settle on a surface or in an environment that can provide nutrients (47). Other results demonstrate that the same factors may be involved in attachment to both types of surfaces (12). Probably, during the development of infections such as subclinical mastitis, biofilm formation could be an efficient way of persisting in the microenvironment of the udder, where shear forces arise during milking (6). The presence of Bap in *S. aureus* strains responsible for subclinical mastitis suggests that Bap may enhance intramammary adherence and biofilm formation, leading to the inefficacy of antibiotic treatment against biofilm bacteria and chronicity. Further studies to demonstrate this hypothesis are warranted.

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