Microbiology (2007), **153**, 1702–1710 DOI 10.1099/mic.0.2006/005744-0

Phase-variable expression of the biofilm-associated protein (Bap) in *Staphylococcus aureus*

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Received 7 November 2006
Revised 8 February 2007
Accepted 20 February 2007

A process of phase variation is described that affects the expression of Bap (biofilm-associated protein) in *Staphylococcus aureus*. Upon subculture of the Bap-positive *S. aureus* strain V329 on Congo red agar, spontaneous smooth biofilm-negative colonies appeared at a low frequency (5×10^{-4}). Northern blot analysis of these variants with a *bap*-specific gene probe showed that transcription of the *bap* gene did not occur. However, DNA typing, Southern blot hybridization and DNA sequencing did not show any differences between the parent V329 strain and the biofilm-negative variants. The biofilm-negative phenotype reverted to wild-type at a similar frequency upon subculture of Bap-negative variants in liquid media. Experimental infection of ovine mammary glands with Bap-negative variants showed that phase variation occurred *in vivo*, because Bap-expressing, biofilm-positive revertants were isolated from infected mammary glands. The absence of Bap correlated with increased adherence to fibrinogen and fibronectin. It is possible that *S. aureus* can detach from a biofilm by switching to a Bap-negative state.

INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for a wide range of both acute and chronic infections. The first step in *S. aureus* infection is adhesion to the extracellular matrix, promoting colonization of host tissues. *S. aureus* can express several adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Foster & Höök, 1998).

Another step in colonization is the formation of a biofilm. Biofilm formation is a major concern in nosocomial infection because it protects microorganisms from opsonophagocytosis and antibiotics, leading to chronic infection and sepsis (Gotz, 2002). Two major surface components have been implicated in biofilm formation by *S. aureus*: (i) the product of the *icaADBC* operon which encodes proteins involved in the synthesis of the polysaccharide intercellular adhesin, the composition of which is poly-*N*-acetylglucosamine (PIA/PNAG; Cramton *et al.*, 1999; Maira-Litran *et al.*, 2002); and (ii) Bap, a surface protein of 2276 aa that contains 13 repeats of 86 residues (Cucarella *et al.*, 2001). Bap promotes both primary attachment to inert surfaces and intercellular adhesion, whereas PIA/PNAG seems to be involved in intercellular adhesion alone.

Interestingly, the *bap* gene is contained in a mobile pathogenicity island (Ubeda *et al.*, 2003), and so far, it has only been found in bovine mastitis isolates (Cucarella *et al.*, 2001) and in isolates of several coagulase-negative staphylococcal species, including *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus simulans* and *Staphylococcus hyicus* (Tormo *et al.*, 2005a). However, to date, none of the human *S. aureus* isolates tested harbours the *bap* gene (Cucarella *et al.*, 2001), suggesting that ruminant and human staphylococcal strains differ in their host-specific pathogenic strategies.

We have previously investigated the relationship between biofilm formation and the functionality of the MSCRAMM proteins in *S. aureus*. Our results have demonstrated that Bap interferes with the activity of the MSCRAMM proteins and decreases: (i) adherence to immobilized fibrinogen and

Abbreviations: FnBP, fibronectin binding protein; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; PIA, polysaccharide intercellular adhesin; PNAG, poly-*N*-acetylglucosamine.

fibronectin; (ii) adherence to mammary gland tissue *ex vivo*; (iii) internalization by epithelial cells; and (iv) initial colonization of the mammary glands of ewes (Cucarella *et al.*, 2002). Similarly, we have observed in a mouse foreign-body-infection model that Bap-deficient mutants colonize catheters more rapidly than does the wild-type at the initial stages of infection, strongly suggesting that the presence of Bap hinders the interaction between bacterial cell receptors and the host proteins on the catheter (Cucarella *et al.*, 2001). However, although initial colonization is affected by Bap, we have demonstrated in several animal models that Bap facilitates long-term persistence of *S. aureus* (Cucarella *et al.*, 2001, 2004; Ubeda *et al.*, 2003).

Phase variation is one of the many strategies employed by pathogenic bacteria to avoid detection by the host immune system. It involves the ability to switch on the expression of proteins when they are needed, and to switch them off when they are likely to trigger immune responses. In *S. epidermidis*, reversible inactivation of the *ica*, *sarA* and *sigB* genes by the insertion sequence IS*256* can result in the production of biofilm-negative variants (Conlon *et al.*, 2004; Ziebuhr *et al.*, 1999). In *S. aureus*, a similar phase-variation process controls expression of the *ica* genes (Valle *et al.*, 2007). However, nothing is known about the role that phase variation might have in the expression of the Bap protein.

In the present study we characterized the phase-variable expression of the Bap protein and demonstrated that this process modulates the functionality of different *Staphylococcus* adhesins.

METHODS

Bacterial strains. Strains used in this study are listed in Table 1. Staphylococcal strains were cultured in trypticase soy agar (TSA) and in trypticase soy broth (TSB), supplemented with glucose (0.25 %, w/v) when indicated.

Screening for biofilm-negative variants. Screening for biofilm-negative variants was performed on Congo red agar (CRA) (Cucarella *et al.*, 2001). Bap-expressing strains grow on this medium as rough colonies, whereas Bap-negative strains exhibit a smooth colony phenotype. Agar plates were incubated overnight at 37 °C, with an additional 24 h at room temperature.

Bacterial adherence to immobilized fibrinogen and fibronectin. Binding of cells to fibrinogen or fibronectin immobilized on plates was measured as described by Hartford *et al.* (1997). Briefly, fibrinogen or fibronectin (Calbiochem) were diluted in sodium carbonate buffer (40 mM, pH 9.6) at the indicated concentration, and 100 µl was used to coat 96-well flat-bottomed ELISA plates (Sarstedt) overnight at 4 °C. Control wells contained carbonate buffer only. After washing with PBS, the plates were blocked for 2 h at 37 °C in 2 % BSA in PBS. Cells from an overnight culture (stationary phase) were washed and diluted in PBS (OD_{600} 1.0). A volume of 100 µl of this cell suspension was added, and the plates incubated for 2 h at 37 °C. After gentle washing, adherent cells were fixed by adding 100 µl 25 % aqueous formaldehyde, and incubating at room temperature for 30 min. The plates were then washed gently, stained with crystal violet, washed again, and read on an ELISA reader at 570 nm.

SDS-PAGE and Western immunoblotting or Western ligand affinity blotting. For ClfA detection, *S. aureus* cells from a stationary-phase culture were suspended to OD_{600} 40 in 100 mM PBS containing 5 mM EDTA and 1 mM PMSF. Cells were centrifuged and suspended in 1 ml digestion buffer (50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$ and 30 % raffinose; Sigma) (Hartford *et al.*, 1997). To each 1 ml sample, 60 µl protease inhibitors (Complete Cocktail; Boehringer Mannheim) and 60 µl 2 mg/ml solution of lysostaphin (Sigma) were then added, and the suspension was incubated in a 37 °C water bath for 30 min. Protoplasts were sedimented by centrifugation at 6000 ***g***, and the supernatant fraction, which contained the wall-associated proteins, was analysed by SDS-PAGE (10 % separation gel, 4.5 % stacking gel).

For Western blot analysis, protein extracts were prepared and analysed by SDS-PAGE as described above, and blotted onto an Immobilon-P membrane (Millipore). Primary anti-ClfA antibody (McDevitt *et al.*, 1994) was used at 1 : 1000 dilution with Tris-buffered saline (TBS; 50 mM Tris/HCl, pH 7.5, 150 mM NaCl) with 1 % skimmed milk. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1 : 10 000 in TBS with 1 % skimmed milk was used, and the subsequent chemiluminescence reaction [with disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo′ [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD, Roche)] was recorded.

The Bap immunoblotting assay was performed as described previously (Cucarella *et al.*, 2001). Briefly, protein extracts were prepared and analysed by SDS-PAGE as described above, and blotted onto an Immobilon P membrane (Millipore). Anti-Bap serum was diluted 1 : 2500 with TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) and immuno-absorbed with 5 % skimmed milk. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1 : 10 000 in TBS/5 % skimmed milk was used, and the subsequent chemiluminescence reaction (CSPD, Roche) was recorded.

For detection of fibronectin binding proteins (FnBPs), the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce) was used to biotinylate

Table 1. Bacterial strains used in this study

Strains	Genotype	Relevant properties	Source or reference
V329	*bap* positive	Expresses Bap protein	Cucarella *et al.* (2001)
m556	V329 *bap* : : Tn*917* Em^r	Deficient in Bap	Cucarella *et al.* (2001)
V329/1–V329/6	*bap* positive	Phase variants of V329; deficient in Bap	This study
V329/rev1	*bap* positive	Revertant strain of V329/1	This study
V329/rev3	*bap* positive	Revertant strain of V329/1	This study
V329/rev2	*bap* positive	Revertant strain of V329/2	This study
V329/rev4	*bap* positive	Revertant strain of V329/2	This study

human fibronectin (Calbiochem). Bacteria grown to OD_{600} 0.2 in 20 ml TSB in a 250 ml Erlenmeyer flask shaken at 200 r.p.m. at 37 °C were harvested by centrifugation at 12 000 ***g***, resuspended, and treated as above. FnBps were detected by Western ligand affinity blotting by incubation for 1 h with biotinylated human fibronectin (50 μg ml^{-1}) in PBS containing 0.1 % Tween 20 (PBST). They were then given three washes in PBST and incubated for 1 h with Streptavidin–POD conjugate (Roche; 1 : 3000 dilution). The membranes were washed as before, and developed using enhanced chemiluminescence (Roche).

DNA methods. General DNA manipulations were performed by standard procedures (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989). Oligonucleotides bap-6m and bap-7c (Cucarella *et al.*, 2001) were used in the Southern and Northern blot experiments.

For Southern hybridization, chromosomal DNA was purified, digested with *Hin*dIII, and separated by agarose gel electrophoresis. Gels were blotted onto nylon membranes (Hybond-N, 0.45 μm pore-size filters; Amersham Life Science) using standard methods (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989). Labelling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labelling and chemiluminescent detection kit (Roche).

RNA extraction was performed according to the method described by Cheung *et al.* (1994). Bacterial cells were grown at 37 °C in TSB to the mid-exponential phase. After harvesting, cell disruption and subsequent RNA extraction were performed by using the FastRNA kit, Blue (BIO 101) and the FP120 FastPrep Cell Disruptor apparatus (Savant Instruments), according to the instructions of the manufacturers.

Forty micrograms RNA of each bacterial strain was applied to a 1.5 % agarose/2.2 M formaldehyde gel in MOPS running buffer. RNA was blotted onto Nylon membranes, UV cross-linked, hybridized with a ^{32}P-labelled *bap* probe in 50 % formamide at 42 °C overnight, washed, and autoradiographed according to standard protocols (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989).

Quantitative assay of biofilm formation on polystyrene. A late adherence assay was carried out essentially as described previously (Cucarella *et al.*, 2001). 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 was used to inoculate sterile, 96-well polystyrene microtitre plates (Iwaki). After 18 h, the wells were gently washed three times with 200 μl sterile PBS, dried in an inverted position, and stained with 0.25 % safranin for 1 min. Wells were rinsed again, and A_{495} was determined (Micro-ELISA Autoreader Elx800; Bio-Tek instruments). Each assay was performed in triplicate and repeated five times.

Experimental infection. The experimental infection was carried out essentially as described previously (Cucarella *et al.*, 2002). Twelve healthy lactating *Rasa Aragonesa* ewes were inoculated with 1 ml bacterial suspension (5×10^2 c.f.u.) 20–25 days after parturition. Mothers were separated from their offspring 2 h before inoculation, to ensure the presence of milk (as a natural lubricant) in the teat duct at the time of inoculation. After teats were disinfected with 70 % ethanol, the inoculum was introduced in both glands through a 21-gauge cannula.

Since suckling favoured removal of bacteria, lambs were separated from their mothers for 120 min, in order to study the capacity of bacteria to colonize the mammary gland.

Milk samples for bacteriological analysis were obtained 2, 5 and 8 days after inoculation. Aliquots of milk samples were placed directly on CRA plates. In addition, to exclude the possibility of contamination, bacteria recovered at the end of the experimental period were compared with the parental strains by DNA typing.

Molecular typing of *S. aureus* strains. Typing of the different strains was performed using three genetic markers: the coagulase, staphylococcal protein A and clumping factor B genes. This combination resulted in a discriminatory power closely approaching that of the whole-genome microarray (Koreen *et al.*, 2005).

PCR amplification of the *coa* gene was performed as described by Hookey *et al.* (1998). Briefly, oligonucleotides coa-1m (5′-ATAGA-GATGCTGGTACAGG-3′) and coa-2c (5′-GCTTCCGATTGTTCG-ATGC-3′), encompassing the entire 3′ repeats were utilized. Each amplification comprised 100 ng DNA template; 100 pmol each primer; 200 μM (each) dATP, dGTP, dCTP and dTTP; 1× buffer (Netzyme); 1 mM $MgCl_2$ and 1 U thermostable DNA polymerase (Netzyme). Water was added to a final volume of 25 μl. Thermal cycling was performed as described (Hookey *et al.*, 1998). An initial denaturation step at 94 °C for 2 min was followed by 30 cycles of 94 °C for 20 s, 57 °C for 15 s and 72 °C for 30 s, with a final step at 72 °C for 5 min. The size of the PCR products (5 μl aliquot) was analysed by electrophoresis on 1 % (w/v) agarose gels.

Restriction endonuclease analysis of the PCR-amplified *coa* gene was performed as described (Hookey *et al.*, 1998). Approximately 500 ng PCR product was digested with 5 U restriction endonuclease *Cfo*I (Roche) at 37 °C for 2 h. The digested PCR product was analysed by electrophoresis on 2 % (w/v) agarose gels.

PCR amplification of the *spa* gene was performed as described by Frenay *et al.* (1994), with modifications. Briefly, the oligonucleotides spa-1m (5′-GATTTTAGTATTGCAATACATAATTCG-3′) and spa-2c (5′-CCACCAAATACAGTTGTACCG-3′) were utilized. An initial denaturation step at 94 °C for 4 min was followed by 40 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 90 s, with a final step at 72 °C for 5 min. The size of the PCR products was analysed by electrophoresis on 1 % (w/v) agarose gels. Restriction endonuclease analysis of the PCR-amplified *spa* gene was performed to increase the power of discrimination, as described for the *coa* gene. In both cases, to validate the patterns obtained after *Cfo*I digestion, the different *coa* and *spa* PCR-amplified products were sequenced, searching for *Cfo*I sites. In all cases, the observed patterns were identical to those predicted after DNA analysis.

PCR amplification of the *clf*B gene was performed as described by Koreen *et al.* (2005).

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

RESULTS

Isolation and frequency of biofilm-negative variants

To investigate the possibility of phase variation, the biofilm-forming Bap-expressing strain *S. aureus* V329 was grown on CRA to isolate biofilm-negative variants. On CRA, Bap-expressing *S. aureus* grows as rough colonies, whereas Bap-negative variants are smooth (Cucarella *et al.*, 2001). In three different experiments, a single colony of the wild-type strain was diluted, grown in liquid culture to exponential phase, and plated onto CRA plates. Ten smooth colonies were picked (Fig. 1a) and tested for

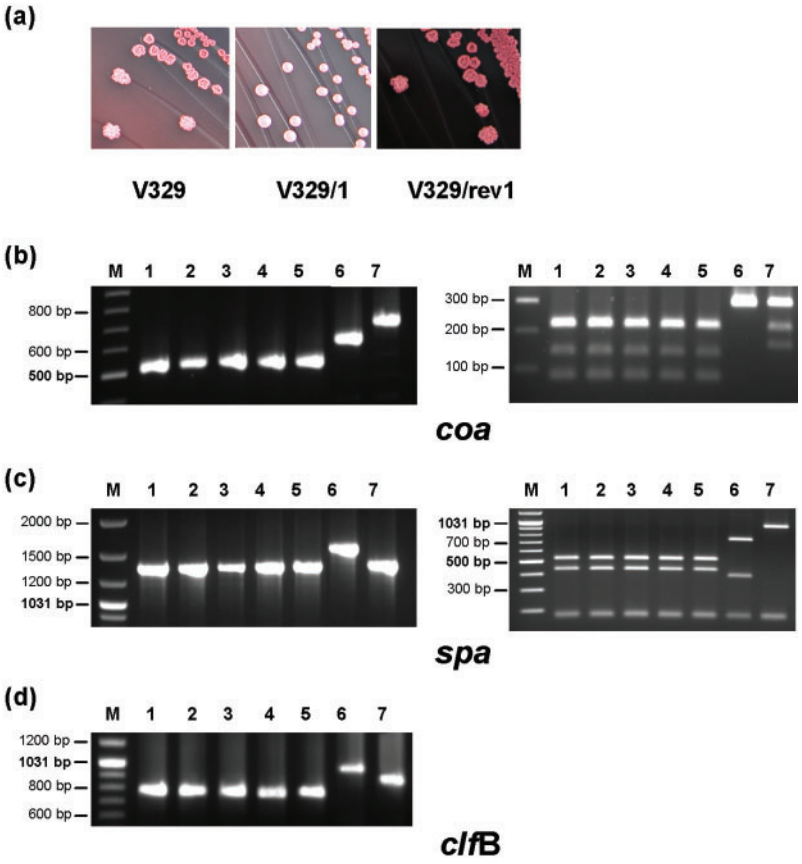

Fig. 1. (a) Phenotypic differences on CRA among the wild-type strain V329, the V329/1 variant, and its derivative revertant V329/rev1. Identical results were obtained with the other V329 variants and revertants (data not shown). (b–c) PCR amplification of the coagulase (b) or protein A (c) genes (left); RFLP electrophoretic patterns of PCR-amplified genes digested with *Cfo*I (right). (d) PCR amplification of the *clfB* gene. Positions of DNA size markers (bp) are shown at the left of each panel. Lane M, molecular mass marker (gene ruler 100 bp DNA ladder plus; Fermentas); lane 1, V329; lane 2, V329/1; lane 3, V329/rev1; lane 4, V329/2; lane 5, V329/rev2; lane 6, Newman; lane 7, N315. Lanes 6 and 7, molecular typing of two laboratory strains used as controls.

biofilm formation on polystyrene tissue-culture plates. All the Congo red-negative variants were biofilm negative (data not shown). The frequency of stable biofilm-negative colonies ranged from 4×10^{-4} to 6×10^{-4}. The wild-type strain and its biofilm-negative variants were analysed by DNA typing, which revealed no detectable difference between the parental strain and its variants (Fig. 1b–d).

Characterization of the biofilm-negative variants

To analyse the relationship between the production of Bap and the spontaneous appearance of biofilm-negative variants, the expression of the Bap protein was determined by Western blotting using anti-Bap polyclonal antiserum. Fig. 2 shows the absence of Bap in the biofilm-negative variants V329/1 and V329/2. Identical results were obtained with all the biofilm-negative variants (data not shown). Since biofilm formation in strain V329 is Bap-dependent (Cucarella *et al.*, 2001, 2004), the biofilm-negative phenotype can be explained by the absence of Bap protein.

As the *bap* gene is contained in a mobile pathogenicity island (Ubeda *et al.*, 2003), we hypothesized that biofilm-negative variants might be generated by the deletion of the element. To investigate this, chromosomal DNA of biofilm-negative variants was tested by Southern blot hybridization using the *bap* gene as a probe. This indicated that the *bap* gene was present in each of the biofilm-negative variants (Fig. 3). Therefore, the lack of Bap

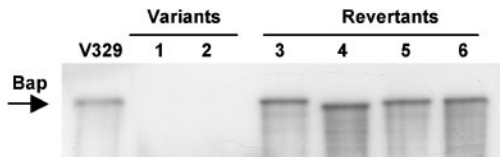

Fig. 2. Western blot analysis with anti-Bap serum. V329, wild-type strain; lane 1, V329/1; lane 2, V329/2; lane 3, V329/rev1; lane 4, V329/rev3; lane 5, V329/rev2; lane 6, V329/rev4.

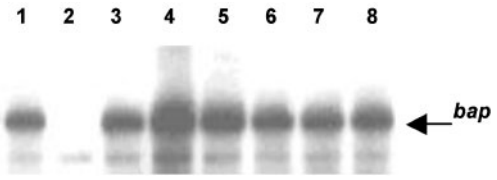

Fig. 3. Southern blot analysis of *Hin*dIII-digested chromosomal DNA with a *bap*-specific DNA probe. Lane 1, V329; lane 2, RN4220 (*bap* negative); lanes 3–8, V329 variants 1–6, respectively.

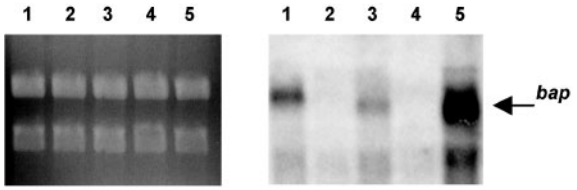

Fig. 4. Northern blot analysis of *bap* transcription. Lane 1, wild-type V329; lane 2, variant V329/1; lane 3, revertant V329/rev1; lane 4, variant V329/2; lane 5, revertant V329/rev2.

expression cannot be explained by the absence of the *bap* pathogenicity island. Similarly, PCR amplification of segments of the *bap* gene failed to reveal any differences between the wild-type and the variants (data not shown).

Finally, we tested whether the absence of Bap in the biofilm-negative variants was due to a defect in transcription of the *bap* gene. Northern blot hybridization with a *bap*-specific probe revealed that the transcription of the *bap* gene was significantly reduced in the Bap-negative phase variants (Fig. 4). To determine if a mutation in the promoter was responsible for the decreased transcription, the *bap* promoter region of various biofilm-negative variants was amplified by PCR and sequenced. There was no modification in the sequence of this region (data not shown), suggesting that decreased transcription might be due to changes in a controlling element.

Selection and analysis of biofilm-positive revertant strains

To address the question of whether the biofilm-negative mutants can revert to a biofilm-positive phenotype, single colonies of *S. aureus* V329/1, V329/2, V329/3 and V329/4 were picked, and biofilm-positive revertants identified. Biofilm-positive revertants were obtained from *S. aureus* V329/1 and V329/2, at a frequency similar to that described for the occurrence of biofilm-negative variants (Fig. 1, Table 2). However, we were unable to obtain revertants from strains V329/3 or V329/4 (Table 2), suggesting that different mechanisms are involved in creating biofilm-negative variants. Revertant strains V329/rev1–V329/rev4 (Table 1) expressed Bap and produced biofilm at the same

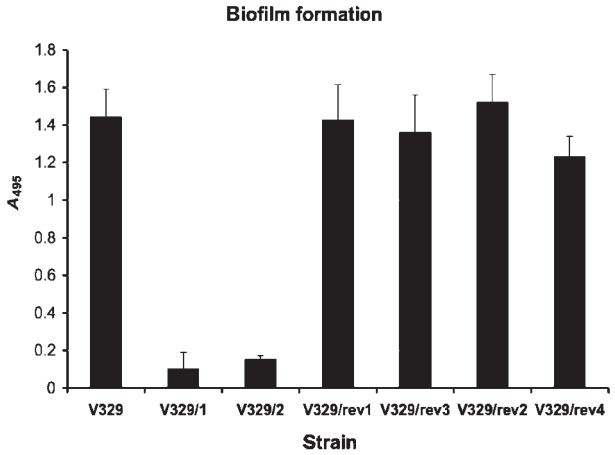

Fig. 5. Biofilm formation *in vitro*. The strains were grown overnight in polystyrene microtitre wells in TSB supplemented with 0.25 % glucose. The cells that adhered to the plate after washing were then visualized by staining with safranin.

level as the wild-type strain (Figs 2 and 5), although they exhibited different levels of *bap* transcription relative to that in the wild-type strain (Fig. 4).

Absence of Bap expression enhances *in vitro* adherence to fibrinogen and fibronectin

To study the influence of the absence of Bap on bacterial interactions with fibrinogen and fibronectin, the wild-type *S. aureus* strain V329, the *bap*-mutant m556, the biofilm-negative variants V329/1 and V329/2, and their corresponding V329/rev1 and V329/rev2 revertants were tested for adherence to immobilized proteins. As previously described (Cucarella *et al.*, 2002), the Bap-mutant m556 cells showed a significantly higher fibrinogen and fibronectin adherence than did the wild-type strain V329 (Fig. 6). Interestingly, the variant V329/1 and V329/2 cells adhered at a higher level to fibrinogen and fibronectin than did the wild-type V329 and its derivative V329/rev1 and V329/rev2 revertant strains (Fig. 6).

To exclude the possibility that increased adherence to fibrinogen or fibronectin in the variants might be due to an increase in the expression of the corresponding

Table 2. Reversion frequency of the biofilm-negative V329 variants

Strain	No. of tested colonies	No. of biofilm-positive revertants	Frequency of reversion*
V329/1	10 800	2	1.9×10^{-4}
V329/2	10 800	3	2.8×10^{-4}
V329/3	83 000	0	$<1.2 \times 10^{-5}$
V329/4	88 000	0	$<1.1 \times 10^{-5}$

*Defined as no. of biofilm-positive revertants/no. of tested colonies.

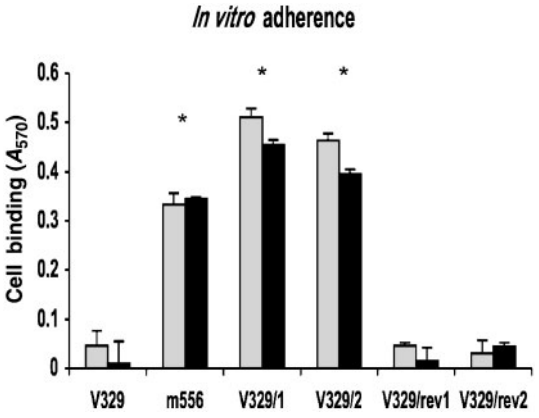

Fig. 6. Adhesion of *S. aureus* V329, m556 (*bap* mutant), V329/1, V329/2, V329/rev1 and V329/rev2 to immobilized fibrinogen (grey bars) or fibronectin (black bars). Data are the mean ± SD of triplicate determinations of the increase in bacterial adherence to uncoated versus protein-coated surfaces. *$P<0.001$, *t* test.

MSCRAMM proteins, we measured by Western blotting or Western ligand affinity blotting, ClfA and FnBPA and FnBPB in strains V329, m556, V329/1, V329/2, V329/rev1 and V329/rev2. As shown in Fig. 7, the different strains produced similar amounts of ClfA and FnBP. These results indicate that the increased adherence to fibrinogen or fibronectin in the Bap-negative strains is not due to higher levels of expression of the MSCRAMMs.

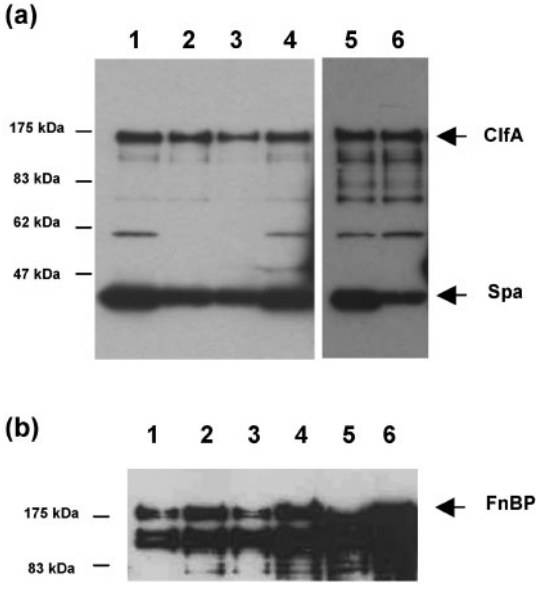

Fig. 7. Visualization of ClfA protein (a) and FnBPs (b) by Western blotting and ligand affinity blots, respectively. Positions of protein size markers (kDa) are shown at the left of each panel. Bands corresponding to native ClfA, FnBPs and Spa proteins are identified. Similar amounts of ClfA, FnBPs and protein A released into the supernatant by protoplasts stabilized in raffinose were observed in the different analysed strains. Lane 1, V329; lane 2, m556; lane 3, V329/1; lane 4, V329/rev1; lane 5, V329/2; lane 6, V329/rev2.

Bap expression is restored *in vivo*

We performed experimental mammary gland infection in ewes to investigate whether phase variation occurs *in vivo*. Six sheep were infected with the biofilm-positive V329 strain and six with the biofilm-negative variant V329/1. Milk samples were taken and plated on CRA. Bap-positive revertants appeared in the animals infected with the V329/1 variant (Table 3). The rough colony revertants were biofilm positive (data not shown). In contrast, we were unable to isolate biofilm-negative variants from sheep infected with wild-type V329. These results contradict the similar frequencies of phase variation observed in both directions *in vitro*. One possible explanation is that increased adherence of Bap-negative variants to the epithelium of the mammary gland gives a selective advantage to hide the Bap-negative variants. Alternatively, it is also likely that environmental conditions biased phase variation toward the Bap-positive phenotype, which favoured persistence in the mammary gland, as previously described (Cucarella *et al.*, 2004; Ubeda *et al.*, 2003). This increased persistence in the Bap-positive isolates could explain the apparently higher reversion observed *in vivo*, particularly at later time points (Table 3).

DISCUSSION

The surface protein Bap is implicated in the formation of biofilms by *Staphylococcus* spp. isolated from chronic mastitis (Cucarella *et al.*, 2001; Tormo *et al.*, 2005a). The presence of Bap reduces infectivity in the short term by blocking early adherence of MSCRAMMs to host proteins (Cucarella *et al.*, 2002). In contrast, Bap plays the opposite role in late adherence, allowing longer bacterial persistence in the mammary gland, most likely through the formation of biofilms (Cucarella *et al.*, 2001; Ubeda *et al.*, 2003). Despite the advantages that bacteria in biofilms have over their planktonic counterparts, dispersal from the biofilm allows sessile bacteria to colonize new niches. In this study, we reported that Bap-negative bacteria arose spontaneously at a high frequency *in vitro*. Phase variation is a strategy used by many pathogenic bacteria to avoid the immune system (Hacker, 1992; Seifert & So, 1988; Weiser *et al.*, 1996). Alternatively, as Bap-negative bacteria have lost the capacity to produce biofilms, phase variation might represent a mechanism for dispersal of *S. aureus* from biofilms.

The synthesis of a large protein like Bap is presumably strictly regulated and coordinated with the production of other elements of the biofilm matrix. A recent study has shown that the global virulence regulator SarA acts as an activator of *bap* expression (Trotonda *et al.*, 2005). SarA can modulate gene transcription through direct interaction

Table 3. Reversion frequency of the biofilm-negative V329 variants *in vivo*

The percentage of Bap-positive strains in the mixed population is shown. Milk samples from the left (L) or right (R) mammary gland were plated on CRA and the percentage of rough colonies was calculated. ND, Not determined.

Animal	Infected with:	2 days post-inoculation		5 days post-inoculation		8 days post-inoculation	
		L	R	L	R	L	R
I	V329/1	Smooth	Smooth	2 % rough	<5 % rough	15 % rough	ND
II	V329/1	Smooth	Smooth	5–10 % rough	5 % rough	100 % rough	80 % rough
III	V329/1	Smooth	Smooth	ND	ND	ND	100 % rough
IV	V329/1	0.5 % rough	0.4 % rough	5 % rough	80 % rough	95 % rough	95 % rough
V	V329/1	50 % rough	Smooth	<1 % rough	50 % rough	40 % rough	90 % rough
VI	V329/1	Smooth	5 % rough	70 % rough	ND	50 % rough	5 % rough

with the target gene promoter, or by activating the *agr* regulatory cascade (Cheung & Zhang, 2002). In the case of the *bap* gene, gel shift and DNase I footprinting assays have shown that SarA directly binds to the *bap* promoter region (Trotonda *et al.*, 2005). Also, disruption of *agr* does not have any effect on *bap* transcription, indicating that the mechanism by which SarA regulates *bap* is *agr* independent (Trotonda *et al.*, 2005). Apart from transcriptional regulation, the function of Bap is subject to modulation by Ca^{2+}. The presence of calcium in the growth medium has an inhibitory effect on biofilm formation (Arrizubieta *et al.*, 2004). The concentrations of Ca^{2+} responsible are similar to those of free Ca^{2+} in milk. The interaction of Bap with Ca^{2+} occurs through EF-hand domains, since disruption of two of the four putative EF-hand motifs results in a Bap protein that is able to induce biofilm formation, even in the presence of Ca^{2+} (Arrizubieta *et al.*, 2004). We now propose an alternative strategy to regulate the function of Bap, based on the ON-to-OFF phase variation of Bap expression. The mechanisms underlying phase variation are unknown. There is no evidence for the involvement of global regulators such as *sarA*, *agr* and σ^B, since the expression of surface adhesins ClfA, ClfB, FnBPA and FnBPB, the expression of which is dependent on these regulators, does not change in Bap-negative variants (Cheung *et al.*, 2004; Novick, 2003).

A mechanism of phase variation affecting biofilm development has been described in *S. epidermidis*, and involves reversible insertion/excision of the IS*256* in the *icaADBC* operon (Ziebuhr *et al.*, 1999). The *icaADBC* operon encodes proteins responsible for the synthesis of the PIA/PNAG exopolysaccharide, which is an important component of the staphylococcal biofilm matrix (Cramton *et al.*, 1999; Maira-Litran *et al.*, 2002). More recently, it has been reported that IS*256* can cause biofilm-negative variants in *S. epidermidis*, through insertion/excision in the *sarA* and *rsbU* genes (Conlon *et al.*, 2004), which are positive regulators of the *ica* operon (Knobloch *et al.*, 2004; Tormo *et al.*, 2005b). The appearance of biofilm-negative variants was significantly more frequent than reversion to biofilm production. In the case of Bap phase variation, similar frequencies in either direction occurred during growth *in vitro*. However, the frequency of Bap-positive variants seems to be higher than the reverse during infection of the mammary gland, suggesting that phase variation responds to environmental conditions. In contrast, Baselga *et al.* (1993) have analysed phase variation in slime production of *S. aureus*, and observed that the severity of ruminant mastitis decreases, but the bacterial capacity to colonize the mammary gland increases, when the infection is caused by a mucoid (slime producer) rather than non-mucoid variant. Samples from the glands infected with the biofilm-negative variant (strain c104−) revealed that none of the 180 500 colonies on CRA showed a biofilm-positive morphology, indicating that reversion from biofilm non-production to production had not taken place. However, glands infected with the biofilm-positive strain c104+ revealed biofilm-negative variants in three animals (in five of the six infected glands of these animals). Reversion was observed at 24 and 48 h after inoculation, suggesting that it occurred immediately after bacterial inoculation. We know that the mucoid strain used in that study (c104+) carries the *bap* gene (Ubeda *et al.*, 2003), while the non-mucoid variant c104− is *bap* negative, since it has lost SaPIbov2. This explains the fact that no biofilm-positive (*bap*-positive) isolates were obtained from the biofilm-negative variants. In view of these results, we used here strain V329, in which the SaPIbov2-integrase gene, responsible for the deletion of the island, is not functional (Ubeda *et al.*, 2003).

The biofilm structure may depend on the nature of the molecules involved. BLAST searches (Altschul *et al.*, 1997) for sequences homologous to Bap have shown the existence of a novel family of proteins, which are important for biofilm formation in both Gram-positive and Gram-negative bacteria (Lasa & Penades, 2006). Members of this family have been described in *S. aureus* (Cucarella *et al.*, 2001), coagulase-negative staphylococci (Tormo *et al.*, 2005a), *Enterococcus faecalis* (Esp; Shankar *et al.*, 1999; Toledo-Arana *et al.*, 2001), *Burkholderia cepacia* (Bap; Huber *et al.*, 2002), *Pseudomonas putida* (mus20; Espinosa-Urgel *et al.*, 2000) and *Salmonella typhimurium* (Stm2689;

Latasa *et al.*, 2005). All members of the Bap family share similar characteristics, including a high molecular mass, a signal sequence for secretion, and a core domain of repeats, the number of which varies among different isolates (Shankar *et al.*, 1999) and during the course of infection (Cucarella *et al.*, 2004). We have not checked whether expression of these proteins is phase variable, but this could be an interesting avenue of further enquiry.

ACKNOWLEDGEMENTS

M. Á. T. and C. Ú. contributed equally to this work. This work was supported by grant BIO2005-08399-C02-02 from the Comisión Interministerial de Ciencia y Tecnología (CICYT) and grants from the Cardenal Herrera-CEU University, the Conselleria de Agricultura, Pesca i Alimentació (CAPiA), and the Generalitat Valenciana (ACOMP06/235) to J. R. P. T. J. F. acknowledges the Science Foundation Ireland (grant 03/IN3/B370) and the Health Research Board (RPO09/2002).

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Edited by: J. H. Cove