

Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer?

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The biofilm-associated protein (Bap) is a surface protein implicated in biofilm formation by *Staphylococcus aureus* isolated from chronic mastitis infections. The *bap* gene is carried in a putative composite transposon inserted in SaPIbov2, a mobile staphylococcal pathogenicity island. In this study, *bap* orthologue genes from several staphylococcal species, including *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus simulans* and *Staphylococcus hyicus*, were identified, cloned and sequenced. Sequence analysis comparison of the *bap* gene from these species revealed a very high sequence similarity, suggesting the horizontal gene transfer of SaPIbov2 amongst them. However, sequence analyses of the flanking region revealed that the *bap* gene of these species was not contained in the SaPIbov2 pathogenicity island. Although they did not contain the *icaADBC* operon, all the coagulase-negative staphylococcal isolates harbouring *bap* were strong biofilm producers. Disruption of the *bap* gene in *S. epidermidis* abolished its capacity to form a biofilm, whereas heterologous complementation of a biofilm-negative strain of *S. aureus* with the Bap protein from *S. epidermidis* bestowed the capacity to form a biofilm on a polystyrene surface. Altogether, these results demonstrate that Bap orthologues from coagulase-negative staphylococci induce an alternative mechanism of biofilm formation that is independent of the PIA/PNAG exopolysaccharide.

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INTRODUCTION

Coagulase-negative staphylococci (CNS) are a group of adaptable and opportunistic pathogens whose ability to persist and multiply in a variety of environments causes a wide spectrum of diseases in both humans and animals. In humans, infection by *Staphylococcus epidermidis* (the prototypical species of this group) is one of the most devastating complications of prosthetic joint surgery (Rupp & Archer, 1994). In contrast, in lactating female animals, CNS have

been considered minor pathogens of bovine mastitis. However, many studies have recently shown the importance of CNS infections in the mammary gland of bovines and other species, such as milking goats and sheep (Burriel & Dagnall, 1997; Deinhofer & Pernthaner, 1995; Jarp, 1991). CNS infections are usually milder than those produced by *Staphylococcus aureus*, which is a major contagious pathogen of bovine mastitis. CNS-infected mammary tissues exhibited greater leukocyte infiltration and increased connective tissue stroma over an uninfected control (Trinidad *et al.*, 1990). One study showed that CNS infections caused an 8.7% loss in milk production from a 305-day milk-yield total (Timms & Schultz, 1987).

Even though more researchers have realized the importance of CNS intramammary infections, the virulence factors of

Abbreviation: CNS, coagulase-negative staphylococci.

The GenBank/EMBL/DDBJ accession numbers for the *bap* sequences reported in this paper are DQ008303 (*S. chromogenes*), DQ008304 (*S. xylosus*), DQ008305 (*S. simulans*), DQ008306 (*S. epidermidis*) and DQ008307 (*S. hyicus*).

CNS remain poorly understood. The virulence factors of bovine or ovine staphylococci have been studied most extensively in *S. aureus*. In this species, many chronic infections are associated with bacterial growth in the form of adherent colonies surrounded by a large exopolysaccharide matrix, constituting a biofilm (Cucarella *et al.*, 2004). Given their aggregate size, biofilms are not susceptible to macrophage phagocytosis, and they become resistant to certain antibiotics (Amorena *et al.*, 1999; Cucarella *et al.*, 2004).

The implication of biofilms in chronic infections has triggered an increasing interest in the characterization of genes involved in biofilm formation (Caiazza & O'Toole, 2003; Gotz, 2002; Lim *et al.*, 2004; Tormo *et al.*, 2005; Valle *et al.*, 2003). In a previous study, we identified a surface protein (Bap, for Biofilm associated protein) implicated in *S. aureus* biofilm formation (Cucarella *et al.*, 2001). 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). The presence of Bap significantly increased the ability of organisms to colonize and persist in the bovine mammary gland *in vivo*. At the same time, Bap-positive isolates were less susceptible to antibiotic treatments when forming biofilms *in vitro* (Cucarella *et al.*, 2004). Analysis of the structural *bap* gene revealed the existence of alternative forms of the Bap protein, which contain a different number of repeats, in *S. aureus* isolates obtained under field conditions throughout the animal's life. The presence of anti-Bap antibodies in serum samples taken from animals with confirmed *S. aureus* infections indicated the production of Bap during infection (Cucarella *et al.*, 2004). Altogether, these results demonstrate that the presence of Bap in the bovine intramammary gland may facilitate a biofilm formation that is connected with the persistence of *S. aureus*. Interestingly, although Bap was previously identified in bovine mastitis *S. aureus* isolates, a similar protein called Bhp (Bap homologue protein; AY028618) is present in human strains of *S. epidermidis*, where it may have a function similar to that of Bap.

The purpose of this study was to investigate whether CNS from mastitis are able to form biofilms *in vitro*, and, if so, whether biofilm formation is mediated via the Bap protein. Not only did we find that the function of the Bap protein is conserved between *S. aureus* and *S. epidermidis*, but also that the *bap* gene is present in several other *Staphylococcus* species, implying that the primary attachment, as well as the cell-cell adhesion function mediated by Bap, may be important virulence factors for several species of this genus.

METHODS

Bacterial strains, plasmids and culture conditions. A total of 131 CNS strains isolated from bovine, ovine or caprine mastitis were used: 38 *S. epidermidis*, 23 *Staphylococcus xylosum*, 18 *Staphylococcus chromogenes*, 20 *Staphylococcus hyicus*, 8 *Staphylococcus caprae*, 6 *Staphylococcus intermedius*, 6 *Staphylococcus simulans*, 3 *Staphylococcus cohnii*, 3 *Staphylococcus warneri*, 3 *Staphylococcus capitis* and 3

Staphylococcus haemolyticus. The representative strains of this collection are shown in Table 1. For convenience and easier reading of the paper, we have included *S. hyicus* in the CNS group, although it is coagulase positive. Bacteria cultured from the milk samples were identified as CNS by standard procedures (Kloos & Schleifer, 1975), including Gram staining, catalase and coagulase tests, and API STAPH (BioMerieux), and they were stored at -80°C until use.

Escherichia coli DH5 α cells were grown either in Luria-Bertani broth or on Luria-Bertani agar (Pronadisa) with the appropriate antibiotics. Staphylococci were grown in Trypticase soy broth (TSB; Difco) or on Trypticase soy agar (TSA; Difco), and supplemented with glucose (0.25%, w/v) as required. Antibiotics were used at the following concentrations: chloramphenicol, 20 $\mu\text{g ml}^{-1}$; and ampicillin, 100 $\mu\text{g ml}^{-1}$.

Biofilm formation. Biofilm formation on abiotic surfaces was quantified essentially as described elsewhere (Heilmann *et al.*, 1996a), with the following modifications. Staphylococci were grown overnight at 37°C in TSB. The culture was diluted at 1:40, in TSB-glucose, and 200 μl of this cell suspension was used per well to inoculate sterile 96-well polystyrene microtitre plates (Iwaki). After 18 h incubation at 43°C , the wells were gently washed three times with 200 μl sterile PBS, air-dried in an inverted position, and stained with 0.1% safranin for 30 s. The 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.

DNA manipulations. Routine DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989). Plasmid DNA from *E. coli* and staphylococci was purified with a Genelute Plasmid Miniprep Kit (Sigma) according to the manufacturer's protocol, except that the staphylococcal cells were lysed with lysostaphin (12.5 $\mu\text{g ml}^{-1}$; Sigma) at 37°C for 1 h before plasmid purification. Plasmids were introduced into staphylococcal strains by protoplast transformation, using a previously described method (Götz & Schumaker, 1987; Götz *et al.*, 1983). 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 (12.5 $\mu\text{g ml}^{-1}$) at 37°C for 1 h before DNA purification. Chromosomal DNA digested with *Hind*III was analysed by agarose gel electrophoresis. Gels were blotted onto nylon membranes (Hybond-N 0.45 μm pore-size filters; Amersham Life Sciences) using standard methods (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989). The probes corresponding to both the *ica* operon and the *bap* gene were generated by PCR using the following oligonucleotides. The *ica* primers (*ica*-A, 5'-GCCTATTTATTGACAGTCGCTAC-GAA-3'; and *ica*-B, 5'-CGTGTGCTTTAAGCCATTGAAT-3') were designed to amplify part of the *icaA* and *icaB* genes of the *icaADBC* locus (U43366). Primers *sasp*-6m (5'-CCCTATATCGAAGGTGTA-GAATTGCAC-3') and *sasp*-7c (5'-GCTGTTGAAGTTAATACTGT-ACCTGC-3') were used to amplify the *bap* gene (AF288402). The PCR products of the amplified *bap* and *ica* genes were used as DNA probes. Labelling of the probes and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labelling and chemiluminescent detection kit (Roche).

Cloning of the *bap*-encoding genetic element from CNS. Initial PCR analysis using specific primers (*sasp*-6m and *sasp*-7c) for the *bap* gene of *S. aureus* amplified a 0.97 kb fragment from different species of CNS. These different reaction products were directly cloned into the TOPO-PCR system (Invitrogen), and then sequenced. An outward-directed PCR was performed using a Vectorsite II kit (Sigma-Genosys), according to the manufacturer's instructions, to obtain the flanking region to these initial sequences. This system is used to amplify regions of unknown DNA sequence

Table 1. Bacterial strains and plasmids

Strain or plasmid	Species	Relevant properties	Source or reference
RN4220	<i>S. aureus</i>	Restriction-deficient mutant of 8325-4	Kreiswirth <i>et al.</i> (1983)
V329	<i>S. aureus</i>	<i>bap</i> -positive strain	Cucarella <i>et al.</i> (2001)
Newman	<i>S. aureus</i>	<i>icaADBC</i> -positive, biofilm-negative strain	Duthie & Lorenz (1952)
RP62A	<i>S. epidermidis</i>	<i>icaADBC</i> -positive, biofilm-positive strain	Christensen <i>et al.</i> (1982)
Tü3298	<i>S. epidermidis</i>	<i>icaADBC</i> -negative, biofilm-negative strain	Kies <i>et al.</i> (2001)
C532	<i>S. epidermidis</i>	<i>bap</i> -positive, biofilm-positive strain	This study
C533	<i>S. epidermidis</i>	<i>bap</i> -positive, biofilm-positive strain	This study
C534	<i>S. epidermidis</i>	<i>bap</i> -positive, biofilm-positive strain	This study
JP39	<i>S. epidermidis</i>	Derivative of C533, <i>bap</i> -mutant, biofilm-negative strain	This study
C427	<i>S. epidermidis</i>	<i>icaADBC</i> -positive, biofilm-positive strain	This study
C482	<i>S. xylosus</i>	<i>bap</i> -positive strain	This study
C483	<i>S. chromogenes</i>	<i>bap</i> -positive strain	This study
12	<i>S. hyicus</i>	<i>bap</i> -positive strain	This study
ATCC 1362	<i>S. simulans</i>	<i>bap</i> -positive strain	Thumm & Götz (1997)
JP40	<i>S. epidermidis</i>	Tü3298(pCU1)	This study
JP41	<i>S. epidermidis</i>	Tü3298(pJP16)	This study
JP42	<i>S. aureus</i>	Newman(pCU1)	This study
JP43	<i>S. aureus</i>	Newman(pJP16)	This study
Plasmids			
pBT2		Shuttle plasmid	Brückner (1997)
pCU1		Shuttle plasmid	Augustin <i>et al.</i> (1992)
pJP15		Vector for recombination experiments; 1.2 kb PCR fragment of <i>bap</i> subcloned in pBT2	This study
pJP16		Vector for complementation experiments; a PCR fragment containing <i>bap</i> from <i>S. epidermidis</i> C533 cloned in pCU1	This study

flanking a region of known DNA sequence. Briefly, the target DNA was digested with an appropriate restriction enzyme. Vectorette units were ligated onto the ends of the cleaved target DNA. PCR amplification was carried out with one primer directed to the known sequence (custom primer), and with the other primer specific for the Vectorette unit (Vectorette primer). The amplified products were then cloned into the TOPO-PCR system (Invitrogen), and they were then either sequenced or used as probes in Southern hybridization experiments.

To verify that the deduced sequence of *bap* represented the native gene without any additions or deletions while manipulating the various clones, sequence information was verified by PCR amplification of select regions of the genes and restriction mapping.

PCR. PCR was performed with a Techne Progene thermocycler in a volume of 25 µl, using DyNAzyme EXT as recommended by the manufacturer (Finnzymes). Cycling times were regulated according to the properties of the primer pairs.

DNA sequencing and computer analysis. The nucleotide sequence was determined by the dideoxy chain-termination method, using an ABI 377 model automatic sequencer (PE Biosystems) at the IBMCP-UPV DNA Sequencing Service (Valencia, Spain). Nested deletions were generated (Erase-a-base system; Promega) for C-repeat sequencing. Similarity searches were carried out using the BLAST 2.0 program (Altschul *et al.*, 1997) on the NCBI server. The cloned sequence was compared against GenBank and the publicly available *S. aureus* genome sequences (TIGR, The University of Oklahoma, and The Wellcome Trust Sanger Institute).

Disruption of *bap*. For disruption of *bap* in the *bap*-positive biofilm-forming isolate *S. epidermidis* C533, an internal 1280 bp

PCR fragment within the N-terminal region of the *bap* gene amplified with primers *bap*-6mP (5'-AAACTGCAGAACAACCAGACAA-ATCATC-3') and *sasp*-3cB (5'-GGGGGATCCCAACCTCGTCAATGGTTAAGTCAGC-3') was cloned into the *Pst*I/*Bam*HI sites of the shuttle vector pBT2 (Brückner, 1997), and the resulting plasmid (pJP15) was introduced into *S. epidermidis* C533 by protoplast transformation (Götz & Schumaker, 1987; Götz *et al.*, 1983). After transformation, transformed bacteria were incubated for 16 h at 32 °C on TSB with chloramphenicol. Subsequently, tenfold serial dilutions of this culture in sterile TSB were plated on TSA with chloramphenicol, and incubated for 24 h at 43.5 °C. After overnight incubation, colonies were analysed for disruption of the *bap* gene by PCR with primers *bapepi*-1mK (5'-CTCTACACAAGTGA-TTCGGTACCTATC-3') and pBT2-1m (5'-TACCCAGGCGT-TTAAGGGC-3'), and the results were confirmed by Southern blot analysis. JP39 represents the insertional *bap*-mutant strain used in this study.

Complementation studies. To prove that the biofilm-deficient phenotype of the mutants was due to the disruption of *bap*, *S. aureus* Newman and *S. epidermidis* Tü3298 were complemented with plasmid pCU1 (Augustin *et al.*, 1992) or pJP16. Plasmid pJP16 carries a PCR-amplified fragment from the wild-type *S. epidermidis* C533 strain, including the *bap* gene under the control of its own promoter cloned in pCU1 (Augustin *et al.*, 1992). Primers *chrom*-6mB (5'-CGCGGATCCTGTTATTTTCATCTATTGGG-3') and *bapori*-2cS (5'-ACGCGTCCGACCCACCTTTGTAAGTGGAGAGCC-3') were used to amplify the *bap* gene from *S. epidermidis* C533. Plasmids pCU1 and pJP16 were transformed into staphylococci via protoplast transformation (Götz & Schumaker, 1987; Götz *et al.*, 1983) or electroporation (Cucarella *et al.*, 2001). Phage 85 was used

to transduce the plasmids from RN4220 to the Newman strain. Stable expression of Bap was analysed in total bacterial extracts by Western blot analysis of proteins run on SDS-PAGE gels.

Sequence-analysis programs. The predicted amino acid sequence of Bap was analysed with the PrositeScan program (Combet *et al.*, 2000) on the web server http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html, in order to identify potential functional domains.

Western blot analysis. The Bap immunoblotting assay was performed as previously described (Cucarella *et al.*, 2002). Briefly, staphylococcal cells from a stationary-phase culture were suspended to an OD₆₀₀ of 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 MgCl₂ and 30% raffinose (Sigma)]. To each 1 ml sample, 60 µl protease inhibitors (Complete cocktail; Roche), and 60 µl of a 2 mg ml⁻¹ 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). Anti-Bap serum (Cucarella *et al.*, 2001) was diluted 1:2500 with Tris-buffered saline (TBS; 50 mM Tris/HCl, pH 7.5, 150 mM NaCl) and immuno-adsorbed with 5% skimmed milk. Alkaline-phosphatase-conjugated protein A (Sigma), diluted 1:10 000 in TBS/5% skimmed milk, was used, and the subsequent chemiluminescence reaction (CSPD; Roche) was recorded.

Statistical analysis. A two-tailed Student's *t* test was used to determine the differences in biofilm formation between the groups. Differences were considered statistically significant when *P* was <0.05.

RESULTS

The *bap* orthologue gene is present in mastitis-derived *S. epidermidis* isolates

We have previously described that the *S. aureus* strains harbouring the *bap* gene are strong biofilm producers. We decided to use the ability to form biofilms in polystyrene microtitre plates as a screening method to determine whether *bap* homologues occur in *S. epidermidis*. For this purpose, 38 *S. epidermidis* isolates from ovine or caprine mastitis, and 50 isolates from human carriers, were tested for their ability to produce biofilms. Only four isolates from animal mastitis displayed a strong biofilm-positive phenotype *in vitro* under our experimental conditions (Fig. 1a). None of the human isolates was a strong biofilm producer. To establish whether Bap plays a role in the biofilm formation process of these isolates, Southern blot analysis, using a specific probe for the *bap* gene of *S. aureus*, was carried out. Interestingly, three out of four biofilm-positive isolates showed a strong hybridization band, suggesting the presence of a *bap*-like gene in these isolates (Fig. 1b). In contrast, we were unable to detect the presence of *bap* in any of the biofilm-negative *S. epidermidis* strains analysed (data not shown).

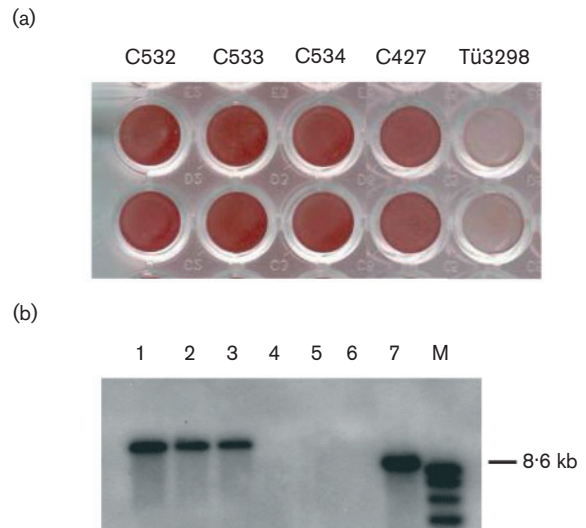


Fig. 1. (a) Biofilm formation in *S. epidermidis* strains. The biofilm-positive *S. epidermidis* strains were grown overnight in polystyrene microtitre wells in TSB supplemented with 0.25% glucose. The cells that adhered to the plate after washing were then visualized by staining with safranin. (b) Southern blot of *S. epidermidis* and *S. aureus* chromosomal digest probed with a *bap* fragment of *S. aureus*. Lanes: 1, C532; 2, C533; 3, C534; 4, C427; 5, RP62A (*icaADBC*-positive *bap*-negative *S. epidermidis* strain); 6, Tü3298 (*icaADBC*-negative *bap*-negative *S. epidermidis* strain); 7, V329 (*bap*-positive *S. aureus* strain); M, molecular mass markers.

Structural analysis of the *S. epidermidis* *bap* gene and deduced protein

The complete *bap* gene of the *S. epidermidis* C533 isolate was cloned and sequenced as described in Methods. An ORF of 8226 bp encodes a protein of 2742 aa residues, with a predicted molecular mass of 284.4 kDa (Fig. 2). An analysis of the Bap primary amino acid sequence from *S. epidermidis* (Bap-e), as previously described for the Bap protein of *S. aureus*, revealed the presence of a typical Gram-positive N-terminal signal sequence for extracellular secretion (first 43 aa; Nielsen *et al.*, 1997), 16 identical 258 nt tandem repeat units encoding reiterations of an 86 aa sequence (C repeats), and a putative C-terminal segment containing a LPXTG motif (Navarre & Schneewind, 1994). A sequence similarity comparison between the Bap proteins of *S. aureus* V329 and *S. epidermidis* C533 is shown in Table 2. The Bap-e sequence also contained five sites with $\geq 80\%$ similarity to the loop of the consensus EF-hand motif.

Deletion of the *bap* gene in *S. epidermidis* abolishes biofilm formation

To further analyse the possible role of the Bap protein in *S. epidermidis* biofilm formation, disruption of the *bap* gene was performed in the biofilm-forming strain C533. For this purpose, a 1.2 kb PCR fragment corresponding to an

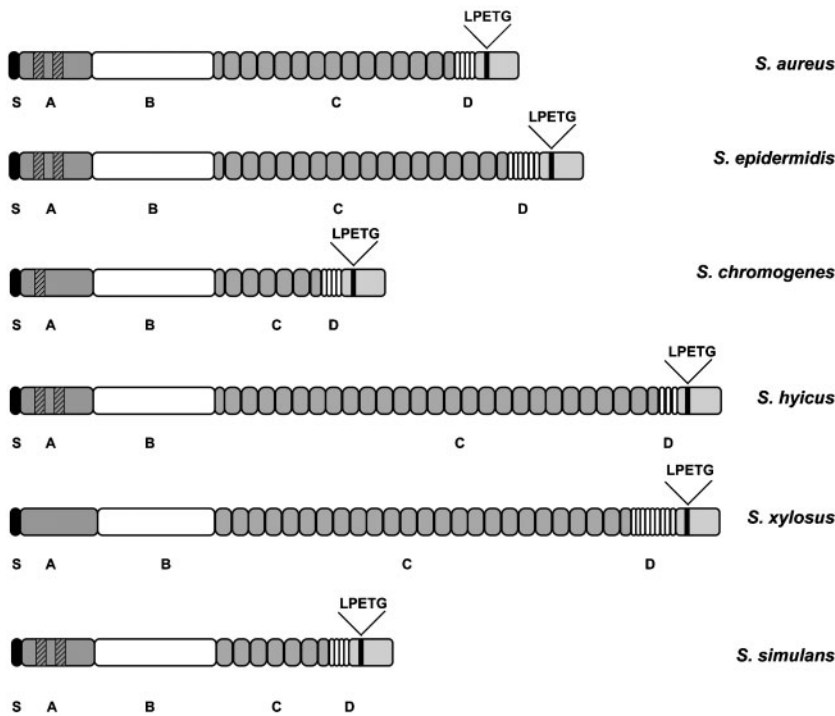


Fig. 2. Structural analysis of the Bap proteins. S represents the signal sequence. The positions of the LPXTG motif, and the A, B, C and D domains, are shown.

internal N-terminal region of *bap* was cloned into the shuttle vector pBT2, producing the plasmid pJP15. The natural biofilm-forming strain C533 was transformed with pJP15, and the transformants were grown in the presence of chloramphenicol at a temperature nonpermissive for plasmid replication. A recombinant isolate, JP39, was selected, and both PCR and Southern blotting confirmed an insertional recombination of the *bap* gene (data not shown). Western blot analysis using anti-Bap polyclonal antiserum confirmed the absence of any hybridization band in the JP39 isogenic mutant, whereas a strong hybridization band of a high molecular mass appeared in the total protein extract of the parental C533 strain (Fig. 3a). To establish a direct connection between the expression of the Bap protein and biofilm formation, the deletion mutant was then

tested for its ability to form a biofilm *in vitro*. As shown in Fig. 3(b), the JP39 mutant was unable to form a biofilm when compared with the wild-type strain ($P < 0.0001$).

Furthermore, complementation experiments were performed to determine whether overexpression of Bap-e bestowed biofilm formation ability upon both *S. epidermidis* and *S. aureus* *bap*-negative strains. For this purpose, plasmid pJP16 harbouring the whole *bap* gene of *S. epidermidis* C533 was introduced into *S. epidermidis* Tü3298 and *S. aureus* Newman, generating JP41 and JP43 respectively (Table 1). The expression of the Bap protein in the complemented strains was confirmed by Western blot (Fig. 4a). In all cases, overexpression of Bap increased the capacity of the complemented strains to form a biofilm on a

Table 2. Sequence comparison of the *bap* locus between staphylococcal species

Species	Size of the predicted polypeptide (aa)	DNA identity (%) [*]	Protein [†]	
			Identity (%)	Similarity (%)
<i>S. aureus</i>	2276	100	100	–
<i>S. epidermidis</i>	2742	97	97	98
<i>S. chromogenes</i>	1530	98	98	99
<i>S. hyicus</i>	3278	97	97	98
<i>S. xylosus</i>	3271	74	80	82
<i>S. simulans</i>	1674	97	97	98

^{*}Sequence comparisons were performed against the *bap* gene from *S. aureus*.

[†]Sequence comparisons were performed against the Bap protein from *S. aureus*.

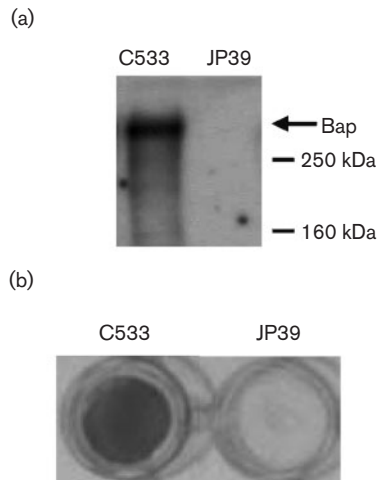


Fig. 3. (a) Analysis of the presence of Bap by Western blotting. A specific band is recognized by polyclonal antibodies against the first 640 aa of Bap in the wild-type *S. epidermidis* strain C533 (lane 1) only, and not in the *bap*-mutant JP39 strain (lane 2). (b) Differences between the wild-type strain C533 and mutant JP39 in the capacity to form a biofilm on polystyrene microtitre plates after staining with safranin.

polystyrene surface (Fig. 4b). Collectively, these results suggest that Bap-e is involved in the biofilm formation process of *S. epidermidis*.

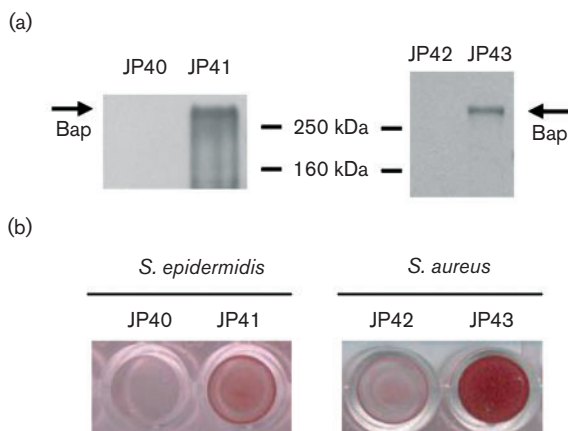


Fig. 4. Complementation studies involving biofilm formation in biofilm-negative strains Newman (*S. aureus*) and Tü3298 (*S. epidermidis*). (a) Western blot analysis of the presence of the Bap proteins in *S. epidermidis* Tü3298 carrying plasmid pCU1 (JP40), *S. epidermidis* Tü3298 carrying pJP16 (JP41), Newman carrying pCU1 (JP42), and Newman carrying pJP16 (JP43). (b) Biofilm formation capacity differences of the different complemented strains correspond to the biofilm formed overnight on polystyrene microtitre plates after staining with 0.1% safranin. Significant differences in adherence ($P < 0.01$) were noted between Bap-expressing and Bap-non-expressing strains.

The *bap* gene is also present in other pathogenic *Staphylococcus* species

The biofilm formation process has also been described as an important virulence factor for different species of CNS. Thus we decided to examine the presence of Bap orthologues in other *Staphylococcus* species by once again using the capacity to produce biofilm *in vitro* as a screening method for *bap*-gene-containing isolates. We analysed the biofilm formation capacity of 93 bovine, ovine and caprine mastitis isolates. Only 13 isolates displayed a strong biofilm-positive phenotype *in vitro* under our experimental conditions (data not shown). A Southern blot containing chromosomal DNA of the selected biofilm-positive isolates was hybridized with *bap* DNA probe from *S. aureus*. Cross-species hybridization was detected with isolates of *S. xylosus*, *S. hyicus* and *S. chromogenes* (Fig. 5a). All the staphylococcal isolates analysed reacted with the antibody raised against the Bap protein of *S. aureus* (Fig. 5b), which is clearly in accordance with the presence of a *bap* gene. However, consistent with the differences observed in the primary sequence, the Bap protein of *S. xylosus* displayed a weaker reaction in comparison with that of other Bap proteins (data not shown). Additionally, all the *bap*-positive isolates were biofilm positive (Fig. 5c).

One representative *bap* gene candidate from each staphylococcal species was cloned, as described in Methods, and then sequenced. Fig. 2 shows a schematic representation of the Bap protein structure from different CNS isolates. A sequence analysis comparison amongst the Bap proteins from the different *Staphylococcus* species is shown in Table 2. The high sequence similarities between the Bap proteins of the different CNS species are worthy of note. One of the most significant differences amongst the Bap proteins of the different CNS species is the number of A, C and D repeats. Thus, two A repeats were contained in the Bap protein of *S. aureus*, *S. epidermidis*, *S. simulans* and *S. hyicus*, whereas only one repeat was present in *S. chromogenes* (Fig. 2). With regard to the C region, the estimated number of C repeats was 16 for the C533 strain (*S. epidermidis*), 25 for strain 12 (*S. hyicus*), 24 for strain C482 (*S. xylosus*), 5 for the C483 strain (*S. chromogenes*), and 6 for strain ATCC 1362 (*S. simulans*). The variation in the number of the C repeats could account for the different mobilities observed in the Western blot experiment (Fig. 5b). Finally, the deduced number of D repeats was 6 for the C533 strain (*S. epidermidis*), 3 for strain 12 (*S. hyicus*), 4 for the C483 strain (*S. chromogenes*), 4 for strain ATCC 1362 (*S. simulans*) and 9 for strain C482 (*S. xylosus*). Interestingly, the sequence and the size of the D repeats in the Bap protein from *S. xylosus* were completely different from those described for the other Bap proteins.

Bap orthologues induce biofilm formation in the absence of PIA/PNAG exopolysaccharide

The biofilm formation process of *S. epidermidis* has been related to the products of the chromosomal intercellular

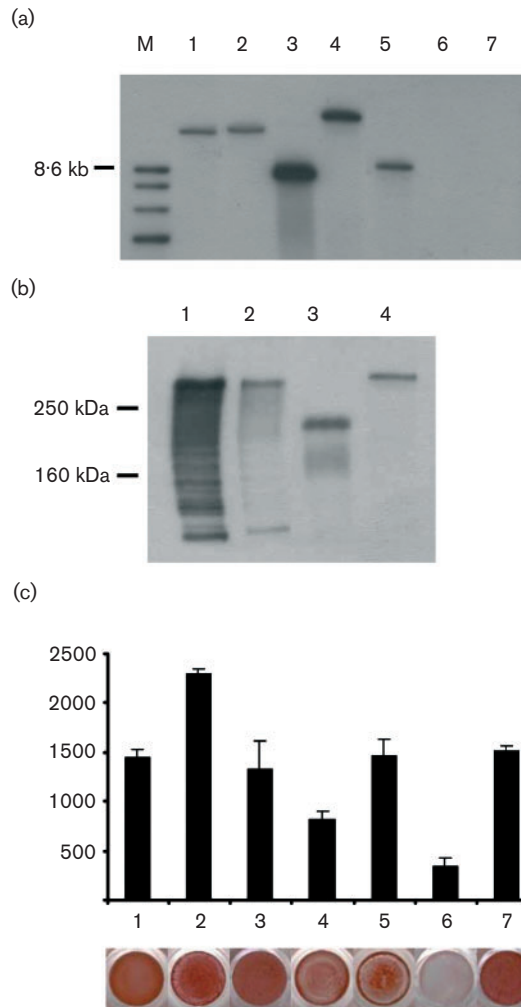


Fig. 5. (a) Southern blot of staphylococcal chromosomal digest probed with a *bap* fragment of *S. aureus*. Lanes: M, molecular marker; 1, C533 (*S. epidermidis*); 2, C482 (*S. xylosus*); 3, C483 (*S. chromogenes*); 4, 12 (*S. hyicus*); 5, V329 (*S. aureus*); 6, RP62A (*S. epidermidis*); 7, Tü3298 (*S. epidermidis*). (b) Western blot analysis of the presence of Bap. Specific bands are recognized by polyclonal antibodies against the Bap protein in *S. aureus* V329 (lane 1), *S. epidermidis* C533 (lane 2), *S. chromogenes* C483 (lane 3) and *S. hyicus* 12 (lane 4). (c) Differences in biofilm formation capacity of the different *bap*-positive strains correspond to the biofilm formed overnight on polystyrene microtitre plates after staining with 0.1% safranin. The bars represent the mean absorbance values at 490 nm (\pm SEM). 1, *S. epidermidis* C533; 2, *S. chromogenes* C483; 3, *S. xylosus* C482; 4, *S. hyicus* 12; 5, *S. simulans* ATCC 1362; 6, *S. epidermidis* Tü3298; 7, *S. epidermidis* RP62A.

adhesion (*ica*) operon. To determine the necessity of the *icaADBC* operon in the biofilm formation process of the *bap*-positive CNS isolates, chromosomal DNA from these isolates was hybridized by Southern blot with an *ica*-specific gene probe. Interestingly, the results revealed the absence of the *icaADBC* operon in all the *bap*-positive CNS analysed

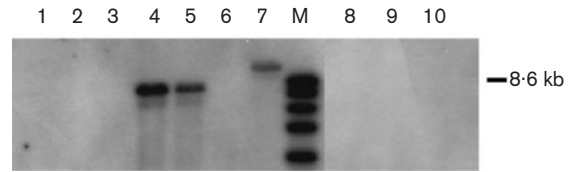


Fig. 6. Southern blot of staphylococcal chromosomal digest probed with an *ica* fragment of *S. epidermidis*. Lanes: 1, C532; 2, C533; 3, C534; 4, C427; 5, RP62A; 6, Tü3298; 7, V329; M, molecular mass markers; 8, C482; 9, C483; 10, strain12.

(Fig. 6). Dot-blot experiments using anti-PIA/PNAG polyclonal antibodies were carried out to confirm the absence of PIA/PNAG-related exopolysaccharides in the CNS isolates, except in *S. xylosus* (data not shown). These results suggest that the Bap protein is able to induce biofilm formation on abiotic surfaces in the absence of PIA/PNAG exopolysaccharide.

A Bap homologue exists in human isolates of *S. epidermidis*

We searched eubacterial genome databases for sequence homologies to the *S. aureus* Bap protein using the gapped BLASTP program (Altschul *et al.*, 1997). Interestingly, in the genome of the strong biofilm former *S. epidermidis* RP62A (<http://www.tigr.org>) we found a protein similar to Bap, which we called Bhp (Bap homologue protein). Analysis of the Bhp primary amino acid sequence revealed the presence of a typical Gram-positive N-terminal signal sequence for extracellular secretion (first 43 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 (Fig. 7). Following the putative signal peptide, the N-terminal region of Bhp (amino acids 46–760) exhibited 20% identity and 35% similarity with the Bap protein of *S. epidermidis*. The central region of Bhp (amino acids 761–2056) begins with a spacer region followed by 14 nearly identical tandem repeat units encoding reiterations of an 89 aa sequence (B repeats; Fig. 7). The B-repeat region begins with a partial B-repeat sequence corresponding to the last 22 aa of a B repeat, and it ends with a partial B-repeat sequence corresponding to the first 28 aa of a B repeat. The B-repeat region accounts for 54% of the Bhp protein, and each repeat shows high sequence identity. BLASTP searches of the B repeats exhibited 31% identity and 44% similarity with the C region of the Bap protein of *S. epidermidis* (Fig. 7). The C-terminal region of Bhp comprises the C region and the LPXTG motif. The C region (amino acids 2077–2221) consists of two repeats of 59 aa separated by 27 aa. These repeats exhibited 53% identity and 65% similarity with the C region of the Bap protein of *S. epidermidis* (Fig. 7). Finally, LPXTG is a consensus

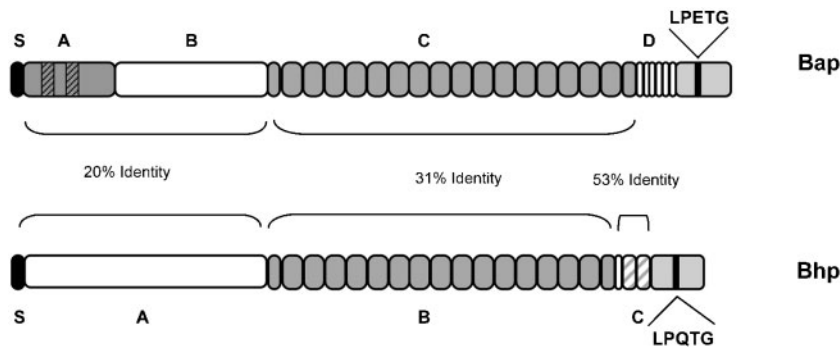


Fig. 7. Structural similarity between Bap and Bhp. The percentage identity between different regions of these proteins is shown. The Bhp structure is derived from strain RP62A. The Bap structure is derived from the *S. epidermidis* C533. The signal peptide (S), the cell-wall anchor (LPXTG motif) and the different regions are shown. The N-terminal domain of Bhp, and regions A–B of Bap, share 20% sequence identity. The B- and C-repeat regions of Bhp, and the C-repeat region of Bap, share 31% and 53% sequence identity, respectively.

cell-wall-anchor motif found in most wall-associated surface proteins of Gram-positive bacteria (Navarre & Schneewind, 1994).

Because of this remarkable structural similarity, it is likely that Bhp could be involved in biofilm formation by *S. epidermidis*. However, no experimental evidence to support this hypothesis has been obtained to date.

The *bap* genes from the different *Staphylococcus* species are not carried by SaPIbov2

In a previous study, we demonstrated that the *bap* gene of *S. aureus* is carried in a putative composite transposon inserted in SaPIbov2, a pathogenicity island of *S. aureus* (Ubeda *et al.*, 2003). The putative transposon harboured the *bap* gene, along with genes encoding an ABC transporter and a transposase. We hypothesized that the *bap* gene might have used the transposon and/or the pathogenicity island mobility to spread among staphylococcal species. To answer this question, we cloned and sequenced the flanking regions of each of the *bap* genes. Interestingly, a sequence analysis of these flanking regions showed that the *bap* genes were not contained in the transposon or in the previously described SaPIbov2 pathogenicity island (data not shown). These results indicate that the presence of *bap* gene in SaPIbov2 is restricted to *S. aureus* isolates, and that it is not the mechanism for horizontal transfer amongst CNS species.

DISCUSSION

The *bap* gene is present in different staphylococcal species

Although the ability of CNS to cause chronic mastitis is well recognized, little is known about the staphylococcal virulence factors that contribute to their pathogenesis. For instance, factors that may influence the ability of staphylococci to colonize host tissues, or their survival in grossly different host environments, are poorly understood. In this study, we have identified and characterized different *bap* genes from infection-derived staphylococcal isolates,

including *S. epidermidis*, *S. chromogenes*, *S. xylosus*, *S. hyicus* and *S. simulans*. The *bap* genes from CNS are involved in biofilm formation, as previously described in *S. aureus* (Cucarella *et al.*, 2001). In *S. aureus*, the presence of Bap has been shown to significantly increase the ability to colonize and persist in the bovine mammary gland *in vivo*. Additionally, Bap-positive isolates are less susceptible to antibiotic treatments when forming biofilms *in vitro* (Cucarella *et al.*, 2004). Animals naturally infected with Bap-positive strains have a lower milk somatic cell count, which implies a reduced severity in the inflammation of the mammary gland, a finding related to the biofilm production capacity in the Bap-positive *S. aureus* strains (Cucarella *et al.*, 2004). If we were to take the sequence identity between the described Bap proteins into account, it is tempting to speculate that all these functions described for the Bap protein from *S. aureus* will be present in the different Bap-positive CNS isolates, suggesting that there is a functional conservation of the biofilm-formation process mediated by the *bap* gene between staphylococcal species from ruminant mastitis.

The prevalence of the *bap* gene itself among mastitis-related isolates analysed was found to change depending on the herd and geographical area. Remarkably, Bap can be found (usually in a small proportion) among mastitis isolates of different species, yet it is absent from the human *Staphylococcus* spp. tested so far (our unpublished results). This difference between the human and ruminant mastitis isolates suggests that these strains are not clonally related, and that specific host-dependent pathogenic factors may have evolved independently in both humans and ruminants. This diversity between isolates from different hosts corroborates the results of Herron *et al.* (2002), who, in a preliminary analysis of the genome of a common clone of bovine *S. aureus*, showed the presence of numerous genes and sequences that differentiate this bovine isolate from previously characterized human *S. aureus* strains. These differences indicate that a rational and effective strategy to control intramammary infections caused by bovine-specific isolates may be advantageous.

We have demonstrated in a previous study that calcium modulates Bap-dependent multicellular behaviour in *S.*

aureus (Arrizubieta *et al.*, 2004). We found that adding millimolar amounts of calcium to the growth media inhibited intercellular adhesion and biofilm formation of the Bap-positive strain V329. Addition of manganese, but not magnesium, also inhibited biofilm formation, whereas bacterial aggregation in liquid media was greatly enhanced by metal-chelating agents. In contrast, virtually no effect of calcium or chelating agents was observed in the aggregation of the Bap-deficient strain m556. Site-directed mutagenesis of two putative EF-hand domains resulted in a mutant strain capable of biofilm formation, but the biofilm of which was not inhibited by calcium. In summary, our results indicated that Bap binds calcium with a low affinity, and that calcium binding renders the protein non-competent for biofilm formation and intercellular adhesion. Interestingly, a motif search of the amino acid sequence of the different Bap proteins using the PROSITE PS00018 definition revealed the presence of these EF-hand domains in the Bap proteins from CNS strains. The presence of the functional EF-hands in the Bap proteins, and the fact that calcium inhibition of Bap-mediated aggregation takes place *in vitro* at concentrations similar to those found in milk serum, support the possibility of this inhibition being physiologically relevant to the pathogenesis and/or epidemiology of the bacteria in the mastitis process.

Bap is the prototype of a new family of surface proteins involved in biofilm formation

The biofilm structure may depend on the nature of the molecules involved in biofilm formation. BLAST searches (Altschul *et al.*, 1997) for sequence homologues to Bap showed the existence of a novel family of proteins, previously named BAP (Biofilm Associated Proteins; Cucarella *et al.*, 2004), which are important for biofilm formation in both Gram-positive and Gram-negative bacteria. Members of this family have been described in *S. aureus* (Bap; Cucarella *et al.*, 2001), CNS (Bap; this study), *S. epidermidis* (Bhp; our unpublished results), *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; McClelland *et al.*, 2001). All members of the Bap family share the following characteristics: (i) a high molecular mass; (ii) a signal sequence for extracellular secretion; and (iii) a core domain of repeats, the number of which varies among different isolates (Shankar *et al.*, 1999) and throughout the course of an infection (Cucarella *et al.*, 2004). Interestingly enough, a variation in the number of repeats in the analysed proteins affected neither the functionality of the protein nor its biofilm formation capacity. These phase-switching differences in the repeat numbers could be related to an evasion of the immune response, as observed in the structurally related alpha C protein (Madoff *et al.*, 1996), which undergoes antigenic variation.

Although proteins belonging to this family have been involved in biofilm formation, their presence is not

absolutely necessary for the process. In this context, *S. aureus* and *S. epidermidis* are fully capable of forming biofilms in the absence of the *bap* gene, as mediated by the *icaADBC* operon (Cramton *et al.*, 1999; Heilmann *et al.*, 1996b), the *hla* gene (Caiazza & O'Toole, 2003), or the expression of a 190 kDa protein regulated by the *rbf* gene (Lim *et al.*, 2004). Additionally, Cramton *et al.* (1999) demonstrated the presence of the *icaADBC* operon in other staphylococcal species, suggesting that the cell-cell adhesion mediating the *icaADBC* operon is highly conserved within this genus. In the same way, others, as well as ourselves, have described that the biofilm formation process of *Ent. faecalis* could be independent of Esp (Kristich *et al.*, 2004; Toledo-Arana *et al.*, 2001). However, it is important to remark that both staphylococci and enterococci can also form biofilms that are exclusively mediated by either the Bap or Esp expression, respectively (Cucarella *et al.*, 2001; Toledo-Arana *et al.*, 2001). Interestingly, the expression of the Bap protein in *Ent. faecalis* enhanced its biofilm-formation capacity (A. Toledo-Arana, unpublished results), confirming the relationship between structure and function between these two proteins. In conclusion, these results suggest that the biofilm formation process in these species is a very complex process mediated by different genes.

Is *bap* transferred between staphylococcal species by horizontal transfer mechanisms?

Horizontal gene transfer is an important source of change in bacteria. In a previous study we described SaPIbov2 (Staphylococcal pathogenicity island bovine 2), which encodes the biofilm-associated protein Bap (Ubeda *et al.*, 2003). SaPIbov2 has an extensive similarity to previously described pathogenicity islands of *S. aureus*. The main difference is that the toxin genes present in the other pathogenicity islands are exchanged for a transposon-like element carrying the *bap* gene, and also genes encoding an ABC transporter and a transposase. Also, SaPIbov2 can be excised to form a circular element, and it can integrate site-specifically and RecA-independently at a chromosomal *att* site depending of Sip, an integrase present in SaPIbov2. Thus, SaPIbov2 encodes a functional recombinase of the integrase family that promotes element excision and insertion/integration. We studied the possibility that transduction can facilitate transfer by taking the high identity between the *bap* genes present in the different staphylococci into account. Interestingly enough, Richard P. Novick (personal communication) has demonstrated that SaPI1, the prototypical pathogenicity island of *S. aureus*, can be transduced from *S. aureus* to CNS. Our preliminary results have shown that SaPIbov2 can also be transduced at a high frequency from *S. aureus* to CNS. This mechanism could explain the high identity observed between the *bap* genes, with the exception of the *bap* gene from *S. xylosus*. However, analysis of the flanking sequences to the *bap* genes present in the CNS led to the rejection of this hypothesis, since the sequences are completely different amongst themselves, and also in comparison with those present in SaPIbov2

(unpublished results). However, it is important to remark that it is possible to identify sequences related to transposases and recombinases near the *bap* gene in our best-characterized *bap*-positive CNS species, *S. epidermidis* (our unpublished results). Additionally, similar structures in members of the Bap family proteins have been described for *esp*, which is flanked by insertion elements (IS) carried into a pathogenicity island of *Ent. faecalis* (Shankar *et al.*, 2002). The roles that these transposon-related sequences and pathogenicity islands play in the mechanism implied in the dissemination of the *bap* gene between staphylococcal species are unknown. We believe that the answers to these questions will clarify the intriguing links between mobile pathogenicity islands, DNA exchange, and the biofilm formation ability of bacteria.

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