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The Gene *bap,* Involved in Biofilm Production, Is Present in *Staphylococcus* spp. Strains from Nosocomial Infections

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This study analyzed ten strains of coagulase-negative staphylococci (CNS) involved in nosocomial infections in three Brazilian hospitals. Their antibiotic susceptibility profile showed that most strains exhibited multiple antibiotic resistance and possessed the *mecA* gene. The ability of these strains to adhere to polystyrene microtiter plates was also tested and nine of them proved to be biofilm producers at least in one of the three conditions tested: growth in TSB, in TSB supplemented with NaCl, or in TSB supplemented with glucose. The presence of the *bap* gene, which codes for the biofilm-associated protein (Bap), was investigated in all ten strains by PCR. All strains were *bap*-positive and DNA sequencing experiments confirmed that the fragments amplified were indeed part of a *bap* gene. The presence of the *icaA* gene, one of the genes involved in polysaccharide intercellular adhesin (PIA) formation, was also detected by PCR in eight of the ten strains tested. The two *icaA*-negative strains were either weak biofilm producer or no biofilm producer, although they were *bap*-positive. To our knowledge, this is the first report demonstrating the presence of the *bap* gene in nosocomial isolates of CNS, being also the first report on the presence of this gene in *Staphylococcus haemolyticus* and *S. cohnii.*

***Keywords*:** nosocomial staphylococci, *bap* gene, biofilm, *Staphylococcus haemolyticus*, *Staphylococcus cohnii*

In the last decade, the nosocomial infections have emerged as the principal cause of mortality in hospitals and the coagulase-negative staphylococci (CNS) are the second bacteria most prevalent in these cases (NNIS, 2004). The importance of these bacteria is due to their role in infections related to implanted medical devices and to the increased frequency of multiple drug resistance among them (Bannerman and Peacock, 2007), which is one of the major public health concerns in the beginning of the 21st century. Moreover, clinical use of novel antimicrobial agents to control bacterial infections will certainly impose new selective pressures and will continue to drive the development of resistance (NNIS, 2004).

The ability of *Staphylococcus* spp. to cause diseases in humans and animals has been attributed to the production of a variety of virulence factors, including biofilm formation. This ability has been described in *Staphylococcus aureus* and in CNS (Deighton *et al*., 1988; Jones *et al*., 1992; Costerton *et al.*, 1999). Biofilm formation occurs through sequential steps in which initial attachment of planktonic bacteria to a solid surface is followed by their subsequent proliferation and accumulation in multilayer cell clusters and final formation of the bacterial community enclosed in a self-produced polymeric matrix (Stoodley *et al*., 2002). Biofilm development is a major concern in nosocomial infections, because it protects microorganisms from opsonophagocytosis and against antibiotics (Costerton *et al.*, 1999; Götz, 2002).

The expression of a biofilm-positive phenotype in staphylococcal strains may depend on the presence of glucose, sodium chloride (NaCl), as well as other environmental conditions. (Christensen *et al*., 1982; Knobloch *et al*., 2001). Two surface components have been implicated in biofilm formation: the production of the polysaccharide β-1,6-*N*-acetylglucosamine, named polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* operon (Götz, 2002) and the biofilm-associated protein (Bap), a surface protein encoded by the *bap* gene, which is essential for biofilm formation in some *Staphylococcus* spp. strains isolated from animal infections (Cucarella *et al*., 2001; Vautor *et al*., 2008). These same authors investigated the presence of the *bap* gene in *S. aureus* isolates recovered from humans and concluded that none of the isolates carried this gene.

The *bap* gene in *S. aureus* has 6,831 bp and the Bap protein shows structural characteristics typical of cell-wall-anchored proteins present in Gram-positive bacteria. The N-terminal domain of the Bap protein contains a signal sequence for extracellular secretion, followed by two separated repeats and, according to the Coiled-Coil prediction program, a dimerization domain can be distinguished. This fact suggests that two Bap molecules belonging to the same or different bacteria could undergo dimerization and thus promote biofilm formation (Latasa *et al*., 2006).

The N-terminal region of Bap can be divided into two regions. Region A (aa 45 to 360) contains two short repeats

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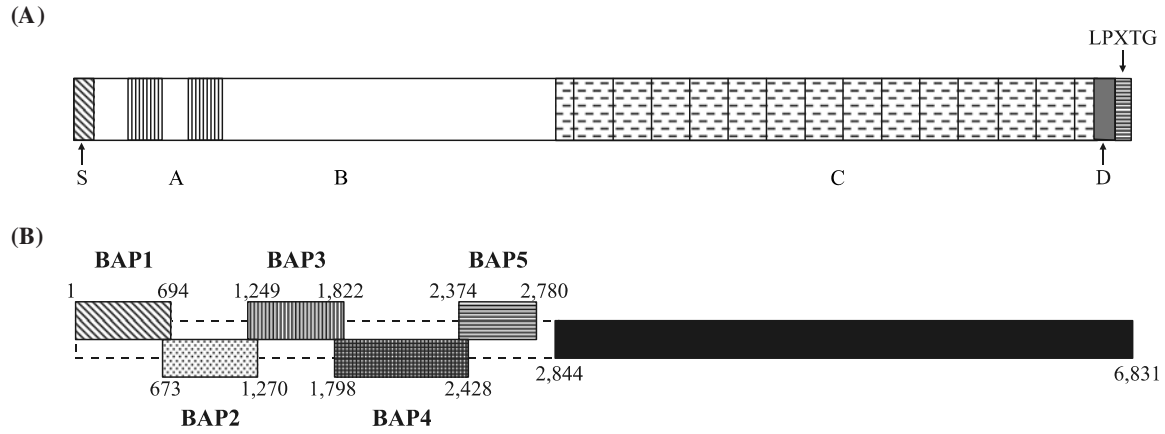

Fig. 1. (A) Bap structure. S represents the signal sequence. The positions of the LPXTG motif and the A, B, C, and D domains are shown. Region A (aa 45 to 360) contains two short repeats of 32 aa (repeats A1 and A2) separated by 26 aa. Region B is the remaining part of the N-terminal domain (aa 361 to 818). 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). The carboxy-terminal region of Bap comprises the D region and the LPXTG motif (adapted from Cucarella *et al*., 2001). (B) The regions of the *bap* gene amplified by the primers used. The numbers indicate the initial and final nucleotides of the fragments. The black bar represents the region of the gene not amplified, including the repeat region, that starts in nucleotide 2,844.

of 32 aa (repeats A1 and A2) separated by 26 aa. 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), 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). The carboxy-terminal region of Bap comprises the D region and the LPXTG motif. The Bap structure is shown in Fig. 1A (Cucarella *et al*., 2001).

The *ica* genes, encoding the biosynthetic machinery for PIA, have been demonstrated to be implicated in the pathogenesis of human staphylococcal infections. However, up to now, Bap has been found only in strains of *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus simulans*, and *Staphylococcus hyicus* isolated from mammary glands in ruminant suffering from mastitis, but no human staphylococcal isolate has been described to date to harbor the *bap* gene (Götz, 2002; Tormo *et al*., 2005). Based on these findings some authors suggest that animal and human staphylococcal strains differ in their host-specific pathogenic strategies (Latasa *et al.*, 2006).

In the present study, CNS strains involved in nosocomial infections in Brazil were isolated and investigated in relation to antibiotic susceptibility and the ability of these strains to form biofilm in polystyrene microtiter plates. Moreover, the strains were tested for the presence of the *bap* and *icaA* genes. The results described in this report clearly demonstrated that the *bap* gene may also be present in clinical

Table 1. Nosocomial CNS strains involved in human infections and control strains

Species	Strain	Source	Resistance profile[a]	Amplification with primers				
				ICAA	BAP1	BAP2	BAP3	BAP4
Nosocomial strains								
Staphylococcus cohnii	38s	Fistula	Gm, Pc, Ox	+	+	+	+	+
Staphylococcus epidermidis	16s	Surgical wound	Cm, Sft, Ap, Gm, Cl, Em, Im, Pc, Mup, Ox, *mecA*$^+$	+	+	+	+	+
	13Hp	Blood	Cl, Em, Pc, Mup, Cip, Ox, *mecA*$^+$	+	+	+	+	+
	18Hp	Blood	Sft, Em, Pc, Mup, Ox, *mecA*$^+$	-	+	+	+	+
	32Hp	Blood	Sft, Gm, Cl, Em, Pc, Mup, Ox, *mecA*$^+$	+	+	+	+	+
	102Hp	Blood	Gm, Em, Im, Cip, Ox, *mecA*$^+$	+	+	+	+	+
	150Hp	Blood	Gm, Cl, Em, Im, Cip, Rf, Ox, *mecA*$^+$	+	+	+	+	+
Staphylococcus haemolyticus	5Hp	Blood	Cm, Sft, AP, Gm, Cl, Em, Pc, Mup, Cip, Ox, *mecA*$^+$	+	+	+	+	+
	36Hp	Blood	Sft, Gm, Cl, Pc, Cip, Ox, *mecA*$^+$	-	+	+	+	+
	69Hp	Blood	Sft, Gm, Cl, Em, Pc, Cip, Am, Ox, *mecA*$^+$	+	+	+	+	+

[a] Chloramphenicol (Cm), sulfamethoxazole/trimethoprim (Sft), ampicillin (Ap), gentamicin (Gm), clindamycin (Cl), erythromycin (Em), imipenem (Im), penicillin (Pc), mupirocin (Mup), ciprofloxacin (Cip), amikacin (Am), rifampicin (Rf), cefoxitin (Cfo), cefalotin, (Cf), ceftriaxone (Cro), and oxacillin (Ox). $^+$ Strains positive in PCR for the *mecA* gene (methicilin resistance). ATCC, American Type Culture Collection. ND, Not determined.

staphylococcal strains.

Materials and Methods

Bacterial strains and culture conditions

Ten strains of CNS, involved in nosocomial infections, were isolated from patients in three hospitals located in the Southeast region of Brazil. These strains were selected among several *Staphylococcus* spp. isolates after species identification, antibiotic susceptibility test and pulse-field gel electrophoresis (PFGE) typing. *S. epidermidis* ATCC 12228 (*ica*$^-$ and *bap*$^-$) was used as a negative control in PCR for amplification of the *icaA* (Arciola *et al*., 2001) and the *bap* genes. *S. epidermidis* ATCC 35984 (*ica*$^+$ and *bap*$^-$) was used as a positive control in PCR for amplification of the *icaA* gene (Arciola *et al*., 2001), while *S. aureus* V329 (*ica*$^-$ and *bap*$^+$) was used as positive control in PCR for amplification of the *bap* gene (Cucarella *et al.*, 2001). The strains used in the present study are described in Table 1.

Escherichia coli DH5α strain was used in cloning experiments (Sambrook *et al*., 1989).

Staphylococcal strains were grown in TSB medium (Difco), at 37°C for 18 h. *E. coli* cells were grown aerobically in LB medium (Himedia) at 37°C for 18 h (Sambrook *et al*., 1989). Staphylococci and *E. coli* were stored in TSB and LB media, respectively, with 40% glycerol (v/v) at -20°C until used.

The media were supplemented with agar at 1.5% (w/v), when required.

Species identification and PFGE typing

Identification of the staphylococcal nosocomial strains to the species level was carried out by Gram staining followed by conventional biochemical tests (Bannerman and Peacock, 2007). PFGE typing with genomic DNA digested with *Sma*I was carried out as described by Nascimento *et al*. (2002).

Antibiotic susceptibility tests

For these tests, the strains were cultured in Mueller-Hinton agar (Oxoid). The tests were performed by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). The following antibiotics (Sensifar) were used: amikacin (30 μg), ampicillin (10 μg), cefalothin (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem(10 μg), mupirocin (5 μg), oxacillin (1 μg), penicillin (10 U), rifampicin (5 μg), sulfamethoxazole/trimethoprim (25 μg), tetracycline (30 μg), and vancomycin (30 μg). All strains were also tested for the presence of the methicillin-resistance gene (*mecA*) by PCR, as described by Santos *et al*. (1999).

Biofilm formation assays

The ability of the ten CNS strains, isolated from nosocomial infections, to attach to and to form biofilm on sterile 96-well polystyrene microtiter plates (TPP® 92096) was tested essentially as previously described (Christensen *et al.*, 1987), with minor modifications. In these tests, the strains were grown in TSB and in TSB supplemented with either 4% NaCl (w/v) or 1% glucose (w/v). The contents of each well were gently aspirated with a multichannel pipette. The fixation step with Bouin fixative was performed for 1 h and the adherent organisms were stained with crystal violet for 1 h. The absorbance at 570 nm was determined in a Microplate Reader Benchmark (Bio-Rad). Each assay was performed in quadruplicate and repeated three times. The interpretation of the results was performed as described by Stepanovic *et al*. (2007), using as negative control the wells inoculated only with broth.

Genomic DNA isolation

Genomic DNA was prepared from overnight cultures of bacteria grown in 10 ml of TSB at 37°C. The cells were centrifuged at 12,100×g for 10 min and washed in 2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7, 8). The washed cells were resuspended in 900 μl of TE buffer and distributed into two microtubes. Twenty microliters of a lysozyme solution (Sigma; 1 mg/ml prepared in double-distilled

Table 2. Primers used in the experiments

	Primers	Region amplified (nt)	Fragment length (bp)
ICAAF	CGATGGGCTCAAGGTGG	-	287
ICAAR	TTCTTTTCGTAGCGACTGTC		
BAP1F	ATGGGAAATAAACAAGGTTTTTTACC	1-694	694
BAP1R	CTTCTTGTTGTTTATCTGGCTC		
BAP2F	GAGCCAGATAAACAACAAGAAG	673-1,270	598
BAP2R	CATGCTCAGCAATAATTGGATC		
BAP3F	GATCCAATTATTGCTGAGCATG	1,249-1,822	574
BAP3R	CACCTTCGATATATGGTAGTAAGTC		
BAP4F	GACTTACTACCATATATCGAAGGTG	1,798-2,428	631
BAP4R	CCTCTGCATTAATTACTTTAGC		
BAP5F	GTTCCTCTTAAAGAAGGTGCAG	2,374-2,780	407
BAP5R	CTAGCTGTTGAAGTTAATACTG-		

water) were added to each bacterial suspension followed by incubation for 1 h at 37°C. Then, 55 μl pronase (Sigma; 1 mg/ml prepared in double-distilled water), 15 μl 20% SDS (w/v), and 10 μl RNAse A solution (Sigma; 10 mg/ml, prepared in double-distilled water) were added and the tubes were incubated at 37°C for 2 h. After incubation, the preparation was treated with 600 μl of phenol/chloroform: isoamyl alcohol (25/24:1) at room temperature. After 15 min, the preparation was centrifuged at 9,200×g for 5 min, at room temperature. The aqueous phase was collected into a new microtube and treated twice with chloroform:isoamyl alcohol (24:1). The aqueous phase was collected and the DNA was precipitated at -20°C for 18 h, by addition of 50 μl of 3 M sodium acetate (pH 7.0) and 1 ml of cold ethanol. The DNA was pelleted by centrifugation at 12,100×g for 30 min, at 4°C. The pellet was washed with 300 μl of 70% cold ethanol (v/v) by centrifugation at 12,100×g for 10 min. The DNA pellet was dried for 10 min, dissolved in 100 μl double-distilled water, and stored at 4°C.

Detection of genes by PCR

The procedure used to detect the *icaA* gene in all strains involved the following steps: DNA extraction, PCR amplification and analysis by agarose gel electrophoresis. Primers specific for amplification of *icaA*, ICAAF, and ICAAR (Table 2), were synthesized based on conserved sequences found in the *icaA* gene of different CNS deposited in GenBank database. The strains and their accession numbers were: *S. epidermidis* (AF500265), *S. saprophyticus* (AF500270), *Staphylococcus condimenti* (AF500266), and *Staphylococcus capitis* (AF500269). These oligonucleotides were expected to amplify an internal fragment of 287 bp. The reactions were done in a 50 μl final volume containing: (i) 50 pmol of each primer; (ii) 5 ng of template DNA; (iii) 200 μM (each) deoxynucleoside triphfosphates; (iv) 2.5 U of Go *Taq* Flexpolymerase (Promega); (v) 3 mM $MgCl_2$, and (vi) 1× reaction buffer (Promega). The thermal cycling consisted of an initial denaturation step at 92°C for 3 min, followed by 30 cycles at 92°C for 1 min, 52°C for 1 min (annealing), and 72°C for 1 min, and a final extension step at 72°C for 5 min. These experiments were performed at least twice. The amplicons were analyzed by agarose gel electrophoresis [1.4% (w/v)], using as molecular-weight markers the 100 bp DNA ladder (Promega).

For detection of the *bap* gene, four different pairs of primers were synthesized, based on the *S. aureus bap* gene sequence deposited in the GenBank database (accession number AY220730). The sequence of the oligonucleotides and the size of the respective fragments amplified are described in Table 2. These PCR reactions were performed at least twice as described above for the *icaA* gene.

For sequencing purposes, one additional pair of primers (BAP5) was designed. All five pairs of BAP primers were designed in order to obtain a final single fragment, which would cover the 5' half of the *bap* gene. For this reason, the amplicons generated in the PCR reactions should overlap (Fig. 1B and Table 2).

Cloning and DNA sequencing

The *bap* amplicons were cloned into the vector pGEM-T-Easy (Promega) according to the manufacturer's recommendations. The transformations were performed into thermocompetent *E. coli* DH5α cells (Sambrook *et al*., 1989). Presence of the insert in the recombinant plasmids was confirmed by digestion with *Eco*RI, which released the insert. The automated sequencing was performed using the ABI Prism 3100 System and the Terminator Chemistry Big Dyes, version 3.1. Computer sequence analyses were per-

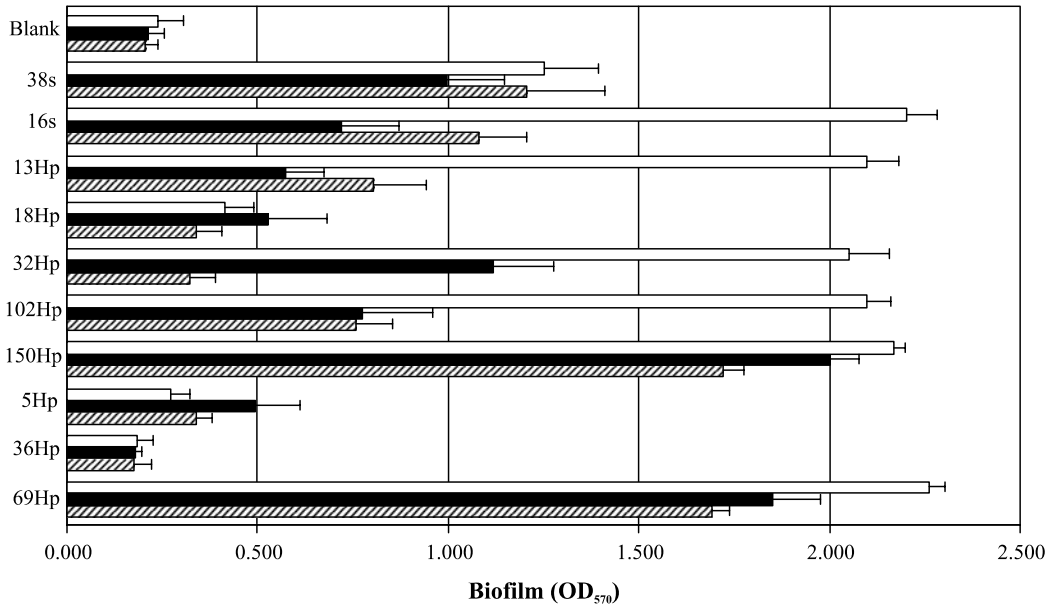

Fig. 2. Bacterial adherence of the staphylococcal strains to the microtiter plates in three conditions tested: growth in TSB (▨), in TSB supplemented with glucose (■) or in TSB supplemented with NaCl (□).

Table 3. Bacterial adherence of the staphylococcal strains to the microtiter plates in three conditions tested: growth in TSB, in TSB supplemented with glucose or in TSB supplemented with NaCl (OD_{570}) and interpretation of the results

Species	Strain	TSB	Biofilm production	TSB+Glucose	Biofilm production	TSB+NaCl	Biofilm production
	Negative control	0.204 (±0.036)	-	0.214 (±0.043)	-	0.239 (±0.066)	-
S. cohnii	38s	1.204 (±0.207)	+++	0.997 (±0.151)	++	1.251 (±0.146)	++
S. epidermidis	16s	1.081 (±0.124)	++	0.719 (±0.142)	++	2.205 (±0.077)	+++
	13Hp	0.806 (±0.136)	++	0.573 (±0.101)	+	2.100 (±0.081)	+++
	18Hp	0.339 (±0.066)	+	0.529 (±0.154)	+	0.414 (±0.074)	-
	32Hp	0.322 (±0.066)	+	1.117 (±0.160)	++	2.051 (±0.104)	+++
	102Hp	0.760 (±0.097)	++	0.776 (±0.184)	++	2.099 (±0.063)	+++
	150Hp	1.721 (±0.055)	+++	2.000 (±0.077)	+++	2.170 (±0.028)	+++
S. haemolyticus	5Hp	0.339 (±0.043)	+	0.496 (±0.115)	+	0.273 (±0.049)	-
	36Hp	0.176 (±0.048)	-	0.181 (±0.018)	-	0.185 (±0.041)	-
	69Hp	1.693 (±0.045)	+++	1.851 (±0.126)	+++	2.262 (±0.039)	+++

-, no production; +, weak production; ++, moderate production; +++, strong production
The numbers represent the means of three independent experiments and the standard deviations.

formed using the program Translator (http://maven.smith.edu). Using the program BioEdit, the predicted amino acid sequences of the putative Bap proteins found in the present work were compared with the amino acid sequences of Bap proteins found in different staphylococcal species involved in bovine mastitis, which were retrieved from the GenBank database.

The DNA sequences reported in this article have been deposited in the GenBank nucleotide sequence database giving the accession number EU011246 (*bap* fragment of *S. haemolyticus*).

Results

In the present study, ten isolates of CNS, most of them from blood, were obtained from patients at three Brazilian hospitals. Their antibiotic susceptibility profile showed that most strains exhibited multiple antibiotic resistance, i.e., resistance to at least three different categories of drugs, and nine possessed the *mecA* gene (Table 1). PFGE typing showed that these ten strains represent different clones (data not shown); so, they were selected for the biofilm experiments.

For easier interpretation of the results related to biofilm production, strains were divided into the following categories: no biofilm producer (–), weak biofilm producer (+), moderate biofilm producer (++), and strong biofilm producer (+++). This classification was possible due to the establishment of a cut-off based on the OD values observed for the negative control (wells containing only broth) as proposed by Stepanovic *et al*. (2007). These results are shown in Table 3.

Nine out of the ten strains were able to adhere to microtiter plates: all the *S. epidermidis* strains, the *S. cohnii* strain 38s, and the *S. haemolyticus* strains 5Hp and 69Hp (Fig. 2 and Table 3). These strains were biofilm producers at least in one of the three conditions tested: growth in TSB, in TSB supplemented with NaCl, or in TSB supplemented with glucose.

The glucose addition did not influence the biofilm production by most of the strains tested, except for strain 32Hp, which had its biofilm production induced by this sugar, and for strains 38s and 13Hp, which seemed to produce less biofilm in the presence of glucose (Table 3).

NaCl addition induced the biofilm production in strains 16s, 13Hp, 32Hp, and 102Hp, but inhibited it in strains 38s, 18Hp, and 5Hp.

The presence of the *bap* gene was tested by PCR using four pairs of primers covering different overlapping regions of the *bap* gene (Fig. 1B and Table 2). Surprisingly, besides the positive control, strain V329, all ten strains tested exhibited amplification in the PCR reactions with the four different pairs of primers (Table 1 and Fig. 3). The amplicons from strain 69Hp are shown in Fig. 4.

To confirm that the fragments amplified were part of the *bap* gene, the amplicons generated by strain 69Hp were cloned into the vector pGEM-T-Easy, sequenced, and assembled in a unique contig, which was then translated. Computer analyses were performed on the partial ORF obtained via the BLASTx program for protein similarity searching.

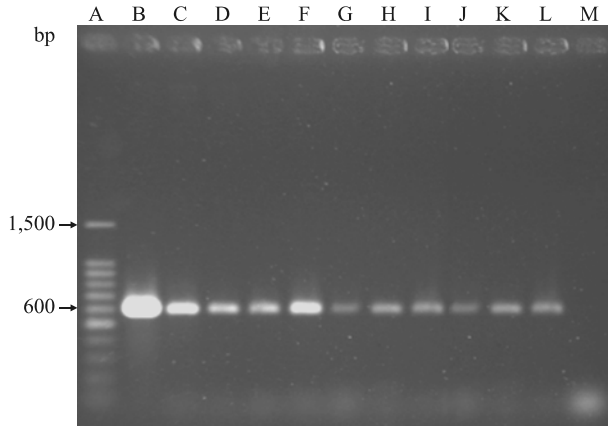

Fig. 3. PCR detection of the *bap* gene with primers BAP2 in nosocomial CNS strains. Lanes A, 100 bp DNA ladder (Promega; some size markers are indicated on the left); B, V329 (positive control); C, 38s; D, 16s; E, 13Hp; F, 18Hp; G, 32Hp; H, 102Hp; I, 150Hp; J, 5Hp; K, 36H; L, 69Hp and M, ATCC 12228 (negative control).

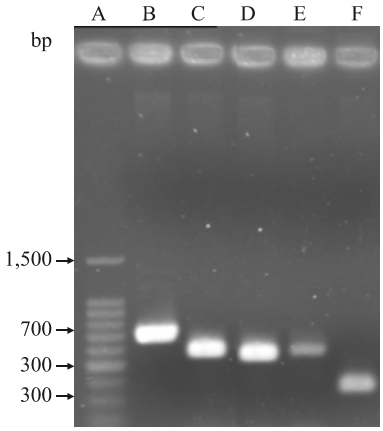

Fig. 4. The *bap* amplicons corresponding to strain 69Hp. Lane A, 100 bp DNA ladder (Promega; some size markers are indicated on the left); B, amplicon from primers BAP1; C, amplicon from primers BAP2; D, amplicon from primers BAP3; E, amplicon from primers BAP4; F, amplicon from primers BAP5.

These analyses revealed a strong similarity between the translated DNA sequence and Bap proteins found in different staphylococcal species, all involved in mastitis. The predicted 926-amino acid sequence encoded by the region sequenced was then aligned with Bap sequences deposited in the GenBank database. The predicted amino acid sequence of the putative Bap protein fragment from strain 69Hp showed 98% identity with the corresponding region of Bap from a *S. aureus* strain (accession number AAK38834), 96% identity with Bap from a *S. epidermidis* strain (accession number AAY28519), 97% identity with Bap from a *S. chromogenes* strain (accession number AAY28516), 96% identity with Bap from a *S. hyicus* strain (accession number AAY28520), and 97% identity with Bap from a *S. simulans* strain (accession number AAY28518), all involved in mastitis (supplementary material). These results confirmed that the fragments amplified and assembled together were indeed part of a *bap* gene.

The presence of the *icaA* gene was also detected by PCR in eight strains (38s, 16s, 13Hp, 32Hp, 102Hp, 150Hp, 5Hp, and 69Hp). Both *icaA*-negative strains, 18Hp and 36Hp, were either a weak biofilm producer or no biofilm producer, respectively (Fig. 2), although they were *bap*-positive (Table 1 and Fig. 3).

Discussion

Coagulase-negative staphylococci are important pathogens involved in nosocomial infections worldwide (Bannerman and Peacock, 2007). With respect to their pathogenic potential, the most important property of CNS is their ability to form biofilm on the surfaces of foreign bodies introduced (implanted) into the patient. In consequence of such formation, they are protected in the biofilm against the effects of antibacterial drugs and of the immune system. Moreover, their acquisition of multiple drug resistance has drastically increased their importance.

In the present report, we have investigated ten nosocomial CNS strains isolated in Brazil, belonging to different PFGE types, in relation to antibiotic susceptibility and the ability to form biofilm. Most strains proved to carry multiple drug resistance and to be biofilm producers at least in one of the three conditions tested. These results are in agreement with Kwon *et al.* (2008), who tested the relationship between multidrug resistance and biofilm formation in *S. aureus* and found a significantly higher rate of biofilm development in strains with greater multiresistance compared with strains with less multiresistance. Infections caused by these strains may pose a serious clinical problem, reinforcing the necessity of getting a better understanding of all mechanisms of biofilm formation by staphylococci.

The glucose addition did not influence the biofilm production by most (70%) of the strains tested. *S. epidermidis* biofilm-positive strains exhibit a PIA- and biofilm-negative phenotype when cultivated in medium lacking glucose, but these strains could be induced to produce PIA by subsequent addition of glucose to the culture medium (Christensen *et al*., 1985). Additionally, the addition of glucose to the culture medium could inhibit, or have no effect on biofilm production by other strains (Christensen *et al*., 1985). However, many authors routinely add glucose directly to the culture medium, aiming to increase the production of biofilm (Ziebuhr *et al*., 1999; Fitzpatrick *et al*., 2005; Araújo *et al.*, 2006). Our present results, together with previous ones, however, show that, before adding the carbohydrate directly to the culture medium, it is advisable to test firstly if the biofilm production by the strains under study is indeed induced by this sugar.

Reports have provided evidence that environmental stresses such as a high osmolarity (high NaCl concentrations) can increase the *ica* operon expression and promote biofilm formation in *S. epidermidis* (Knobloch *et al*., 2001). Indeed, in the present work, the NaCl addition induced the biofilm production by some of the strains tested, but inhibited it in others. To our knowledge, the inhibition of biofilm production by addition of NaCl has not been described in the literature yet. As mentioned for glucose, before adding the NaCl directly to the culture medium, it is advisable to test firstly if the biofilm production by the strains under study is indeed induced by this salt.

The results of detection of the *icaA* and *bap* genes together with the effects caused by addition of either glucose or NaCl to the medium lead us to conclude that there was no relationship between the presence of the genes and the effects of the addition of this carbohydrate or this salt on the capacity of biofilm formation by the strains tested (Table 1 and 3).

Bap is an important surface protein which has been implicated in biofilm formation and is encoded by the *bap* gene (Cucarella *et al.*, 2001). PCR analyses showed that all strains tested in this study seem to carry the *bap* gene. DNA sequence analysis and translation of the 5' half of the putative *bap* gene, covering the 926-amino acid N-terminal region of a Bap protein present in *S. haemolyticus* 69Hp, confirmed the presence of this gene. The region analyzed represents 40.7% of an entire Bap protein, which is generally composed of 2,276 amino acid residues.

Primers for the nucleotide repeat region of the *bap* gene

were not used in the present work, since the presence of these repeats would generate amplicons of different sizes. This complication occurs because the primers may anneal to many similar regions. Therefore, it would be impossible to determine the exact location of each amplicon generated, making difficult the assemblage of the final sequence of the gene by this technique.

Although we have not sequenced the entire *bap* gene of the nosocomial strains, the probability of the presence on the bacterial chromosome of a DNA sequence, coding for a stretch of 926-amino acid residues, not part of a *bap* gene but highly homologous to it, is highly unlikely.

These interesting results show that, in contrast to data described in previous studies (Cucarella *et al*., 2001; Vautor *et al.*, 2008), the *bap* gene seems to be found also in staphylococcal strains isolated from clinical specimens. It is important to note that among the strains which were *bap*-positive, three were identified as *S. haemolyticus* and one, as *S. cohnii.* The *bap* gene has never been found in both species before (Cucarella *et al*., 2001; Tormo *et al*., 2005; Vautor *et al.*, 2008).

PIA is the best-characterized surface molecule involved in staphylococcal intercellular adhesion. In this study, the presence of the *icaA* gene was also detected in most strains tested. With exception of strain 5Hp, all strains which possess both the *icaA* and *bap* genes were strong biofilm producers at least under one of the three conditions tested. In spite of being *bap*-positive, the two *icaA*-negative strains were either a weak biofilm producer or no biofilm producer. These results may suggest that the *bap* gene is probably not being expressed in these strains.

Bap was shown to be essential for biofilm formation in some staphylococcal strains associated to animal infections. The *bap* gene was observed for the first time in a *S. aureus* strain involved in bovine mastitis (Cucarella *et al.*, 2001). It was subsequently detected in CNS also associated with animal infections (Tormo *et al.*, 2005; Latasa *et al.*, 2006).

Therefore, up to the present work, the *bap* gene had never been found in staphylococcal strains isolated from human infections. The fact that the *bap* gene had never been detected in human staphylococci led some authors to suggest that animal and human staphylococcal strains differ in their host-specific pathogenic strategies (Latasa *et al.*, 2006). However, the present results suggest that this seems not to be the case, since the *bap* gene was also found in all nosocomial strains tested.

The *bap* gene has previously been detected only in *S. aureus*, *S. epidermidis*, *S. chromogenes*, *S. xylosus*, *S. simulans*, and *S. hyicus* (Tormo, 2005; Latasa *et al.*, 2006) and, in this study, it has also been found in *S. haemolyticus* and *S. cohnii* strains. Such findings may be attributed to the presence of the *bap* gene in a transposon-like element inserted in a pathogenic island, which is mobile, and to mechanisms of horizontal gene transfer among bacteria (Tormo, 2005; Latasa *et al.*, 2006). Although we have found evidence for the presence of the *bap* gene in human clinical staphylococcal isolates, such finding does not mean that the gene is been expressed. Therefore, further experiments should be performed to investigate if the Bap protein is indeed functional in these microorganisms.

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