Tracking adhesion factors in *Staphylococcus caprae* strains responsible for human bone infections following implantation of orthopaedic material

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Ten *Staphylococcus caprae* strains isolated from four patients and responsible for bone infections following implantation of orthopaedic material were compared to four *S. caprae* strains collected from milk samples of healthy goats. The following characteristics were investigated: *Sma*I patterns, hybridization patterns with pBA2 (ribotypes), slime production, adhesion to matrix proteins (fibrinogen, fibronectin, collagen) and the staphylococcal adhesion genes (*fnbA*, *clfA*, *cna*, *atlE*, *ica*, *fbe*). None of the characteristics enabled us to distinguish the human strains from the goat strains. Slime was occasionally produced by *S. caprae* strains but all of them carried nucleotide sequences hybridizing at low stringency with the following genes: *atlE* encoding a *S. epidermidis* autolysin binding vitronectin and responsible for the primary adhesion to polystyrene, *ica* operon involved in the biosynthesis of a *S. epidermidis* extracellular polysaccharide, and the part of *clfA* encoding the serine-aspartate repeated region of a *S. aureus* cell-wall fibrinogen-binding protein.

Keywords: *Staphylococcus caprae*, bacterial adhesion, autolysin, bone infection, serine-aspartate proteins

INTRODUCTION

It is widely recognized that coagulase-negative staphylococci (CNS), which are major components of the normal flora of the skin and mucous membranes, cause significant clinical infections (Kloos & Bannerman, 1994; Rupp & Archer, 1994). Such infections occur mostly after the implantation of foreign materials and/or in immunocompromised patients. The species that most frequently causes disease in humans is *Staphylococcus epidermidis*, but other coagulase-negative species are increasingly recognized as human pathogens. *Staphylococcus caprae* was originally isolated from goat's milk (hence the name) (Devriese *et al.*, 1983) and is predominant among the CNS recovered

from mastitis-free goat's milk samples (55% of the 165 CNS collected) (Bedidi-Madani *et al.*, 1998a). The CNS of this species are probably widely distributed in human clinical specimens since they accounted for 10.7% of 1230 staphylococci isolated in six Japanese hospitals and identified by DNA–DNA hybridization (Kawamura *et al.*, 1998). The human *S. caprae* strains were reported to have been acquired nosocomially following the administration of antibiotics; these strains carry resistance to several antibiotics, including intrinsic resistance to β -lactams encoded by *mecA* (Kanda *et al.*, 1991; Kawamura *et al.*, 1998). The involvement of *S. caprae* in human disease (Elsner *et al.*, 1998; Shuttleworth *et al.*, 1997; Spellerberg *et al.*, 1998; Vandenesch *et al.*, 1995) has been reported. The routine use of molecular methods to identify staphylococci at the species level (Chesneau *et al.*, 1992, 1993; De Buyser *et al.*, 1989, 1992) led to the detection, in our reference centre, of 10 *S. caprae* strains from four patients suffering from bone and joint infections following the implantation of orthopaedic

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Abbreviations: CNS, coagulase-negative staphylococci; HP, hybridization pattern; MHB, Mueller–Hinton broth; TSB, Trypticase soy broth.

material. The aim of this study was to characterize these ten strains and four unrelated *S. caprae* strains isolated from the milk of mastitis-free goats and, in particular, to track the adhesion factors or genes that have been shown to be implicated in the pathogenesis of staphylococci (Bedidi-Madani *et al.*, 1998a; Christensen *et al.*, 1983; Deighton *et al.*, 1996; Greene *et al.*, 1995; Heinz *et al.*, 1996; Hell *et al.*, 1998; Moreillon *et al.*, 1995; Patti *et al.*, 1994; Rupp *et al.*, 1998; Vaudaux *et al.*, 1993).

METHODS

Patients and analysis of specimens. None of the four patients selected for this study had any contact with goats. Three (patients A, B and C) were infected following the implantation of joint prostheses, whereas the tibia of patient D was infected after osteosynthesis with a plaque and screws (Table 1). The multiple intra-operative specimens (at least three) of patients A, B and D yielded *S. caprae* only. The knee prosthesis of patient C was originally infected with *S. epidermidis*. One month after the end of a treatment with pefloxacin, rifampin and fusidic acid, there was a secondary infection with *S. caprae* in pure culture. In each case, the infected orthopaedic material had to be removed.

For patient A, the total hip arthroplasty was infected and, despite variations in colony morphology and drug-resistance patterns, all the colonies tested belonged to *S. caprae*. After the removal of the prosthesis, the patient was treated for three months with pefloxacin and fusidic acid. A second prosthesis

was implanted five months after the removal of the first and specimens collected during the operation were sterile. The second prosthesis was reinfected with *S. caprae* because of the presence of remaining cement fragments in the distal part of the femur and was removed 19 months after its implantation.

Specimens were cultured on sheep-blood agar for at least 48 h at 37 °C. The isolated colonies with different morphology from each sample were identified at species level as described previously (De Buyser *et al.*, 1989) and tested using the ID32 Staph System (bioMérieux). Patients whose infected specimens contained only *S. caprae* colonies were included in this study. For each patient, the drug-resistance pattern was determined for colonies of *S. caprae* with different morphology. Strains distinguishable by at least one drug-resistance marker were studied independently.

Bacterial strains and plasmids. The relevant characteristics of the *S. caprae* strains isolated from the specimens of each patient and from milk samples are reported in Table 1. The four *S. caprae* strains isolated from milk samples taken from mastitis-free goats consisted of the type strain, CCM 3573^T (Devriese *et al.*, 1983), and three strains previously identified by analysis of their ribotypes (De Buyser *et al.*, 1992): 141-15, CH55 and CH244. The following staphylococcal strains were used as positive controls in adhesion, hybridization and PCR experiments: *S. aureus* Cowan-I (ATCC 12598), *S. aureus* NCTC 8325 (Novick, 1967), *S. aureus* Newman (Duthie & Lorenz, 1952), *S. epidermidis* RP62A (ATCC 35984) (Christensen *et al.*, 1985) and a clinical isolate of *S. epidermidis* A6 (this study) carrying the *fbe* gene (Nilsson *et al.*, 1998). The *S. epidermidis* strain SP2 (ATCC 35982) (Christensen *et al.*,

Table 1. Characteristics of the *S. caprae* strains studied

Abbreviations: As, sodium arsenate; Cd, cadmium acetate; Cm, chloramphenicol; Fm, fosfomycin; Pc, penicillinase production; Sm, streptomycin.

Patient/specimen	Strain designation	Drug-resistance phenotype	ID32 Staph profiles*	Hybridization patterns with pBA2 (Fig. 1)		SmaI genotype (Fig. 2)	Slime production	Size (kb) of HindIII fragment hybridizing at low stringency with:		
				HindIII	EcoRI			attE (K/L)	ica (I/J)	clfA-2 (N/O)
Patient A: Tissues around the two hip prostheses removed during surgery in October 1989	89318	Fm, Pc, Cd, As	367120000†	H802	E804	I	++	3.7	1.8	3
	89319	Fm, Pc, Sm, Cd, As	362000000	H802	E804	I	—	3.7	1.8	3
	89320	Fm, Pc, As	267330000†	H802	E804	I	+	3.7	1.8	3
	91315	Fm, Cd, As	367136400	H802	E804	I	—	3.7	1.8	3
	91316	Fm, As	367136000†	H802	E804	I	—	3.7	1.8	3
	91317	Fm, Pc	367116400	H802	E804	I	+	3.7	1.8	3
	91318	Fm	363114000†	H802	E804	I	—	3.7	1.8	3
Patient B: Tissues around the hip prosthesis, removed during surgery	92375	Fm, Pc, Cd, As	363336000†	H802	E802	II	—	7.0	1.8	4/10
Patient C: Synovial fluid collected during secondary infection	93161	Fm, Pc	363336000†	H800	E805	III	+	7.0	1.8	2.8/3.5/10
Patient D: Tissues around orthopaedic material (screws and plaque, collected during surgery)	96007	Fm, Pc, Cd, As	363136000†	H802	E802	IV	—	8.0	1.8	3.8/11.0
Milk samples from mastitis-free goats	CCM 3573 ^T	Fm, As	265336000†	H800	E800	VI	+	3.7	1.8	2.8/3.5
	141-15	Fm, Pc, Cm, Cd	367136000	H800	E801	VII	—	3.7	1.8	3.75/5/13
	CH55	Fm	367132000†	H800	E801	VIII	+	3.7	1.8	3.7/11
	CH244	Fm, Pc, Cd	365132000†	H800	E801	IX	—	3.7	1.8	3.1

* Software v2.0 updated in May 1998 by bioMérieux was used for identification.

† Consistent with a 94.5 or a 99.9% positive identification of *S. caprae*.

Table 2. Oligonucleotides used for PCR experiments in this study

Primer	Sequence (5'-3')	Gene	Accession no.	Reference	Position
A	GGTAATCATTCATTCGAG	<i>fnbA</i>	J04151	Signas <i>et al.</i> (1989)	2362-2379
B	TGGCACACTGTCGAAGTC	<i>fnbA</i>	J04151	Signas <i>et al.</i> (1989)	2568-2551
C	ATGGTACCAAGAAGATACG	<i>cna</i>	M81736	Patti <i>et al.</i> (1992)	688-705
D	TCTTGATACCAAGCTTGTG	<i>cna</i>	M81736	Patti <i>et al.</i> (1992)	1052-1034
E	GCACCGGCAGCTGGCAC	<i>clfA</i>	Z18852	McDevitt <i>et al.</i> (1994)	974-990
F	GCTCATCAGGTTGTTTCAGG	<i>clfA</i>	Z18852	McDevitt <i>et al.</i> (1994)	1952-1934
G	TAAACACCGACGATAATAACCAAA	<i>fbe</i>	Y17116	Nilsson <i>et al.</i> (1998)	306-329
H	GGTCTAGCCTTATTTTCATATTCA	<i>fbe</i>	Y17116	Nilsson <i>et al.</i> (1998)	801-778
I	GCAGTTACAGGTAATCCACG	<i>ica</i>	U43366	Heilmann <i>et al.</i> (1996)	1235-1254
J	CTCTCTTAACATCATTCCGACG	<i>ica</i>	U43366	Heilmann <i>et al.</i> (1996)	4089-4110
K	CGAAGCAAGTAGCACCTTGG	<i>atlE</i>	U71377	Heilmann <i>et al.</i> (1997)	4142-4161
L	CTTGTGCTGACAAGTATTGTGG	<i>atlE</i>	U71377	Heilmann <i>et al.</i> (1997)	6121-6100
M	GACTTTGAAGAATCTACAC	<i>fnbA</i>	J04151	Signas <i>et al.</i> (1989)	1948-1966
N	GATTAAGCTTTACGTTCAAC	<i>clfA</i>	Z18852	McDevitt <i>et al.</i> (1994)	1805-1824
O	CATTGGTACCATTTTATAGGTG	<i>clfA</i>	Z18852	McDevitt <i>et al.</i> (1994)	2948-2928

1985), which does not produce slime, was used as a negative control. Bacterial suspensions in Brain Heart Infusion containing 15% (w/v) glycerol were stored at -80°C until analysis.

The plasmid pBA2 (Iglesias *et al.*, 1983) was used as a probe. It consists of pBR322 with a 2.3 kb *HindIII* insert containing the *Bacillus subtilis* 16S rRNA gene. pUC18 was used as a cloning vector.

Susceptibility to antimicrobial agents. The pattern of resistance to antimicrobial agents was determined by the disk diffusion method. The markers tested were those used to detect the known staphylococcal drug-resistance phenotypes. Commercially available disks loaded with the following antibiotics were used: penicillin G, oxacillin, spectinomycin, streptomycin, kanamycin, neomycin, gentamicin, tobramycin, chloramphenicol, erythromycin, lincomycin, trimethoprim, sulfonamide, tetracycline, minocycline, pefloxacin, rifampin, fusidic acid, fosfomicin, teicoplanin, vancomycin (Diagnostics Pasteur) and mupirocin (Mast Diagnostics). Additional disks prepared in the laboratory contained 20 µg pristinamycin IIA, 40 µg pristinamycin IB, 0.2 µM cadmium acetate, 0.2 µM sodium arsenate, 0.2 µM mercuric nitrate, 200 µg ethidium bromide, 200 µg acriflavine, 200 µg propamidine isothionate, or 10 µg cetyltrimethylammonium bromide.

DNA isolation and analysis. Total cellular DNA was isolated from staphylococcal strains and was purified using the QIAamp tissue kit from Qiagen. Plasmid DNA was extracted and purified from *Escherichia coli* using the QIAprep spin plasmid kit from Qiagen.

Restriction endonucleases were obtained from Amersham or Pharmacia and were used according to the manufacturer's instructions. Native or digested DNA was analysed by electrophoresis through 0.7% agarose gels. DNA fragments of less than 1 kb, amplified by PCR, were separated by electrophoresis through 4% Nusieve agarose gels (FMC).

Blotting and hybridization. DNA was transferred to Hybond-N+ membranes and hybridized under stringent conditions (68°C) as previously described (Chesneau *et al.*, 1993), or at lower stringency, i.e. 42°C rather than 68°C .

Ribotype determination. Cellular DNA was extracted, cleaved with *HindIII* and *EcoRI* separately, subjected to electrophoresis, transferred onto Hybond-N+ membranes and probed under stringent conditions with pBA2 radio-labelled with [α - ^{32}P]dCTP (110 TBq mmol $^{-1}$) (De Buyser *et al.*, 1989).

The sizes of the bands constituting the hybridization patterns (HPs) were introduced into our database. Each HP was compared with each of the *EcoRI* and *HindIII* HPs previously obtained for validly classified staphylococci (Chesneau *et al.*, 1992; De Buyser *et al.*, 1989, 1992). For each new HP, the percentage similarity to each *EcoRI* or *HindIII* HP in the database was calculated. An isolate with a new HP may be assigned to a known taxon if the highest percentages of similarity obtained are clustered within a single taxon.

PCR. The PCR was performed either at high stringency (initial cycle of 5 min at 95°C followed by 30 cycles of 1 min at 60°C , 1 min 30 s at 72°C and 45 s at 95°C and a final extension step of 10 min at 72°C) or at low stringency (initial cycle of 5 min at 95°C followed by 30 cycles of 1 min at 47°C , 1 min at 72°C and 1 min at 95°C with a final extension step of 5 min at 72°C). The primers used are shown in Table 2, and were prepared by the phosphoramidite method with an Applied Biosystems model 380B DNA synthesizer. They were used to screen for the presence in *S. caprae* strains, of genes carrying homologous sequences (by PCR at high or low stringency), and to amplify, at high stringency, DNA fragments from the sequenced genes to be used as intragenic probes. The oligonucleotides were chosen so as to amplify: (i) almost all of each gene (*ica* with oligos I/J and *atlE* with primers K/L); (ii) the region encoding the part of the protein known to bind the matrix protein (*clfA*-1 with primers E/F, *cna* with primers C/D, *fnbA*-1 with primers B/M and *fbe* with primers G/H) and (iii) the region containing repeats (*clfA*-2 with primers N/O and *fnbA*-2 with primers A/B).

The PCR products used as probes were obtained with the following pairs of primers (Table 2): C/D (365 bp from *cna* of *Staphylococcus aureus* 8325-4), G/H (496 bp from *fbe* of *S. epidermidis* A6), I/J (2876 bp from *ica* of *S. epidermidis* RP62A), K/L (1980 bp from *atlE* of *S. epidermidis* RP62A),

M/B (621 bp from *fnbA-1* of *S. aureus* 8325-4) and N/O (1144 bp from *clfA-2* of *S. aureus* 8325-4).

PFGE of macro-restriction fragments. The protocol for the determination of *Sma*I or *Sst*II restriction patterns was as described previously (Derbise *et al.*, 1996).

DNA sequencing. An Applied Biosystems automated 373A DNA sequencer, and the protocol described by the manufacturer were used for sequencing. The amino acid sequence deduced from the nucleotide sequence was analysed with the GCG package and compared with those deduced from nucleotide sequences in the GenBank/EMBL Database.

Adherence to polystyrene. Adherence to polystyrene, indicating slime production, was quantitatively examined with the spectrophotometric technique as described by Christensen *et al.* (1985). Overnight cultures in Trypticase soy broth (TSB) were diluted 1:100 in fresh TSB and in TSB with glucose (2.5 g l⁻¹). Portions (0.2 ml) were placed in sterile microtitre polystyrene plates (Becton Dickinson Labware). After overnight incubation at 37 °C, the contents of each well were aspirated and washed four times with 0.2 ml PBS (pH 7.2). Adherent organisms were fixed in place with Bouin solution and stained with Hucker's crystal violet. Excess stain was rinsed off with running tap water. After the organisms were dried, the optical densities of the stained adherent bacterial films were measured with a MicroElisa Auto Reader at 570 nm.

The measurement was performed in quadruplicate and repeated twice. The positive control strain was *S. epidermidis* RP62A. Means of the raw data were calculated and transformed by the following formula: adherence = (mean OD₅₇₀ after growth in TSB divided by mean OD₅₇₀ after growth of RP62A in TSB) + (mean OD₅₇₀ after growth in TSB with glucose divided by mean OD₅₇₀ after growth of RP62A in TSB with glucose).

The adherence value obtained for strain SP2 (ATCC 35982) was 0.38. For the other strains tested, slime production was designated +++ for adherence values >2, ++ for values >1 and ≤2, + for values >0.5 and ≤1 and the strains with values ≤0.5 were considered to be non-producers.

Assessment of *in vitro* bacterial attachment to coverslips coated with fibrinogen, fibronectin/gelatin or collagen. Adhesion assays were carried out as described by Greene *et al.* (1995). The strains tested were grown for 5 h in Mueller-Hinton broth (MHB) containing [*methyl*-³H]thymidine. Two-sided coverslips (8 × 9 mm) made of polymethylmethacrylate (PMMA) were coated by incubation for 60 min at 37 °C with solutions containing 0.5, 1 and 2 µg human fibrinogen ml⁻¹ (Chromogenix) or bovine type II collagen (Chemicon). The adsorption of human fibronectin (Chemicon) was optimized by first coating the coverslips with gelatin (1 mg ml⁻¹) (Greene *et al.*, 1995), rinsing them in PBS and incubating them for 60 min at 37 °C with solutions containing 0.25, 0.5 or 1 µg fibronectin ml⁻¹. After incubation of the coated coverslips with 10⁷ c.f.u. [*methyl*-³H]thymidine labelled bacteria ml⁻¹ for 60 min at 37 °C, the number of attached bacteria was estimated from radioactive counts. Uncoated and gelatin-coated PMMA coverslips not exposed to matrix proteins were used as controls. Each dose-response curve was produced from at least two experiments, each including a positive control: *S. aureus* Newman or Cowan-I strains for adhesion to fibrinogen, *S. aureus* Cowan-I for adhesion to fibronectin and to collagen.

Extraction of cell-wall proteins, SDS-PAGE and Western

affinity blotting. Cultures were grown at 37 °C overnight in 50 ml MHB. The overnight culture was then added to 450 ml MHB (OD₆₀₀ ~ 0.1) and incubated for 2–3 h at 37 °C with shaking at 100 r.p.m. Bacteria (OD₆₀₀ ~ 0.3–0.4) were harvested by centrifugation, washed with 20 ml PBS and suspended in 1 ml 1.1 M sucrose in PBS containing 1 mM CaCl₂, 0.5 mM MgCl₂ and 1/25 of a tablet containing a cocktail of protease inhibitors (Complete from Boehringer). Cell-wall proteins were released by treatment with lysostaphin (0.125 mg ml⁻¹) (Ambicin L from Applied Microbiology) at 37 °C for 15 min. The suspension was centrifuged at 12000 g for 10 min, and the supernatant was concentrated and sucrose eliminated on Centricon-100 (Amicon). Protein concentration was determined by the Bradford protein assay (Bio-Rad).

SDS-PAGE was performed using standard procedures (Laemmli, 1970) with a 5% polyacrylamide stacking gel and a 6% resolving gel. Before electrophoresis, 10–30 µl of the solutions containing 10–20 µg protein released from the cell wall were boiled for 5 min in 4× sample buffer (0.25 M Tris/HCl, pH 6.8, 8% SDS, 40%, v/v, glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue).

Proteins were transferred electrophoretically onto PVDF membrane (Hybond-P, Amersham) with a Trans-blot cell blotting system (Bio-Rad). The blotting buffer used was 25 mM Tris/HCl, 192 mM glycine and 15% methanol. Membranes were incubated in TBS blocking reagent (20 mM Tris/HCl, 500 mM NaCl, pH 7.5–8.0) containing 2.5% BSA. Binding proteins were detected by incubating the membranes with 30 µg fibronectin ml⁻¹ for 1 h at room temperature and then with mouse anti-human fibronectin N-terminal monoclonal antibody (Chemicon). Antibodies were detected by chemiluminescence with the ECL kit (Amersham) and anti-mouse IgG-horseradish peroxidase conjugate.

N-terminal amino acid sequence. The blotted proteins released from the cell wall were stained with amido black. The N-terminal amino acid sequence of an excised band of 175 kDa was determined using an Applied Biosystems 473-A sequencer.

RESULTS

Identification at the species level

Using only the ID32 Staph System with a database including *S. caprae* goat strains, only two of the ten human strains, 91316 and 96007 (Table 1), were identified. Taking into account the characteristics of the human strains of *S. caprae* (Kawamura *et al.*, 1998), five of the ten human strains of this study were correctly assigned to *S. caprae* by the same system (Software v2.0 updated in May 1998) (Table 1). This system failed to identify the other five human strains in the hospital laboratory and in our reference centre. The strains isolated from patient A had variable biochemical traits (Table 1).

*Hind*III and *Eco*RI HPs with pBA2 (ribotypes) were determined for the 14 clinical and goat's milk strains reported in Table 1. These strains belonged to five ribotypes: H800–E800, H800–E801, H802–E802, H800–E805, H802–E804 (Table 1). All strains isolated from patient A had the same *Hind*III and *Eco*RI patterns and the patterns of two clinical isolates, 92375 and 96007, were not distinguishable. Schematic repre-

Fig. 1. Schematic representation of the distinct *EcoRI* or *HindIII* hybridization patterns, (a) and (b), respectively, detected among the *S. caprae* strains using pBA2 as probe. The human strains had hybridization patterns H800-E805, H802-E802 or H802-E804 whereas H800-E800 and H800-E801 corresponded to the strains from milk samples.

sentations of each different HP are shown in Fig. 1. The percentage similarities between the *HindIII* or *EcoRI* patterns of the human and goat strains of *S. caprae* were very high ($\geq 73\%$); therefore, the assignment to *S. caprae* on the basis of HP analysis was straightforward.

Drug-resistance patterns

All *S. caprae* strains tested were resistant to fosfomycin (Table 1). Some of the strains carried additional resistance markers: resistance to penicillin by penicillinase production, streptomycin, chloramphenicol, cadmium acetate and sodium arsenate.

Analysis of the *SmaI* macro-restriction patterns

The patterns of the seven strains isolated from patient A were indistinguishable, suggesting that the strains were monoclonal, although isolated 2 years apart (Table 1). The other strains had different genotypes, with differences involving at least seven bands (Fig. 2): they were therefore considered to be polyclonal. This was as expected because no epidemiological links had been found between the patients and/or the goats, which came from different farms.

Although the strains were polyclonal, there were high percentages of similarity between some *SmaI* patterns: 55–78% between the patterns of the four human strains, 51–75% between those of the four goat strains and

Fig. 2. PFGE of *SmaI*-digested DNA from eight *S. caprae* strains. Lane 1, CCM 3573; lane 2, CH244; lane 4, CH55; lane 5, 141-15; lane 6, 93161; lane 7, 92375; lane 8, 89320; lane 9, 96007; lanes 3 and 10, *SmaI*-digested DNA of *S. aureus* strain NCTC 8325 used as standard and lane 11, bacteriophage lambda DNA concatemers (Bio-Rad). The first four *S. caprae* strains (lanes 1, 2, 4 and 5) were isolated from goat milk samples whereas the four other strains (lanes 6–9) were isolated from human specimens.

32–64% between the human and goat strains. The highest percentages were those for similarity between the patterns of the intra-human or intra-goat *S. caprae* strains.

Slime production

Four of the ten clinical *S. caprae* strains and two of the four *S. caprae* strains isolated from milk produced slime (Table 1). Variable results were obtained for the *S. caprae* strains of patient A responsible for the primary and recurrent infections of the hip prosthesis.

In vitro adherence of the *S. caprae* strains to fibronectin/gelatin-, collagen- and fibrinogen-coated coverslips

Regardless of the matrix protein and concentration used to coat the coverslips, the number of *S. caprae* bacteria adhering to each coverslip was much lower than that for the *S. aureus* Newman or Cowan-I strains included as positive controls. The results obtained with fibronectin/

Fig. 3. *In vitro* adhesion to fibronectin/gelatin-coated coverslips (a) and to collagen-coated coverslips (b) of six *S. caprae* strains and *S. aureus* Cowan strain used as a positive control. The *S. caprae* strains are indicated as: ♦, strain 89320; ▲, strain 92375; ■, strain 93161; ○, strain 96007; *, strain 141-15; ●, strain CCM 3573^T. *S. aureus* Cowan is represented by △.

gelatin- and collagen-coated coverslips are shown in Fig. 3. The human isolates of *S. caprae* could not be distinguished from those collected from goat's milk. The level of adhesion was very low but some *S. caprae* strains adhered in a concentration-dependent fashion: strains 89320, 93161, 92375, 141-15 and 96007 with fibronectin/gelatin-coated coverslips (Fig. 3a) and strains 89320 and 93161 with collagen-coated coverslips (Fig. 3b). With fibrinogen-coated coverslips, for each fibrinogen concentration used to coat the coverslips, the ratio of the number of adherent *S. caprae* bacteria per coverslip to that of adherent Newman or Cowan-I bacteria did not exceed 1/38 (results not shown).

Analysis of the proteins released from the bacterial cell wall

The cell-wall proteins of the four *S. caprae* clinical isolates included in this study and the *S. caprae* type strain were screened for binding to human fibronectin (Fig. 4a). An approximately 175 kDa band was repro-

ducibly detected for strains CCM 3573^T, 93161, 92375 and 96007, and an approximately 97 kDa band was present in the patterns of strains 89320 and 96007. Strain 89320 was distinguishable from the other strains by the pattern of proteins stained with Coomassie brilliant blue (Fig. 4b).

The N-terminal part of the ~175 kDa protein released from the cell wall of *S. caprae* strain 96007 (Fig. 4, lane 5) was sequenced. The amino acid sequence obtained was AEETQDQTKN, which is 80% identical to the N-terminal part of *S. aureus* Atl (AETTQDQTTN) (Oshida *et al.*, 1995), 70% identical to the N-terminal part of *Staphylococcus saprophyticus* Aas (ASNTQDQTPN) (Hell *et al.*, 1998) and 50% identical to the same part of *S. epidermidis* AtlE (AEQPQNQSHN) (Heilmann *et al.*, 1997).

Screening for genes encoding staphylococcal adhesion factors

PCR experiments were carried out with primers based on the sequences of the genes *fnbA*, *cna*, *clfA* and *fbe* (Table 2). None of the *S. caprae* strains gave rise to a PCR product at high stringency (60 °C). At lower stringency (47 °C), PCR products were occasionally detected but their sizes were consistently different from those expected if the intact sequenced genes investigated were present and their sequences did not have significant similarities to any of the sequenced adhesion factors (results not shown). We concluded that low stringency PCR was not a productive way of isolating genes for the adhesion factors of *S. caprae*. We therefore decided to use hybridization experiments to detect the presence of genes homologous to *atlE*, *ica*, *fnbA*, *cna*, *clfA* and *fbe*. At low stringency (42 °C), sequences hybridizing to *atlE*, *ica* and *clfA*-2 were detected in all strains tested. The sizes of the *Hind*III fragments hybridizing to these probes are reported in Table 1. Some *S. caprae* strains had several *Hind*III fragments that hybridized at low stringency to the *clfA*-2 DNA encoding the serine-aspartate dipeptide repeat of ClfA.

DISCUSSION

Identification of *S. caprae*

Ecological studies and the recognition of *S. caprae* as a human pathogen have probably been thwarted by difficulties in the precise identification of this species by available phenotypic schemes. Such difficulties in phenotypic identification are not restricted to the human isolates (this study; Kawamura *et al.*, 1998; Shuttleworth *et al.*, 1997; Vandenesch *et al.*, 1995) but are also encountered for goat isolates (Bedidi-Madani *et al.*, 1998b). As illustrated here, there has been a significant improvement in the identification of human *S. caprae* strains by the ID32 Staph System, taking into account the emended description of this species (Kawamura *et al.*, 1998). Resistance to fosfomycin shown by all the strains studied here and by those described by other authors (Elsner *et al.*, 1998;

Fig. 4. SDS-PAGE analysis of the proteins released from the cell walls of *S. caprae* strains: CCM 3573^T (lane 1), 93161 (lane 2), 92375 (lane 3), 89320 (lane 4) and 96007 (lane 5). (a) Fibronectin-binding proteins; (b) proteins stained with Coomassie brilliant blue. The high molecular-mass standard (Bio-Rad) was loaded in lane 6.

Vandenesch *et al.*, 1995) may be a useful presumptive test for the identification of *S. caprae* but several other staphylococcal species, such as *S. saprophyticus*, *Staphylococcus hominis*, *Staphylococcus capitis* and *Staphylococcus auricularis* have natural resistance to this antibiotic (Loulergue *et al.*, 1984).

Although none of the four patients had any direct or indirect contact with goats, none of the phenotypic and genomic characteristics investigated distinguished the human infectious *S. caprae* strains from those isolated from the milk samples of mastitis-free goats. The slight difference between the *Sma*I patterns of human and goat *S. caprae* strains reported by George & Kloos (1994) was also detected for the strains of this study but neither *Sma*I patterns nor ribotypes gave clear-cut discrimination.

Molecular methods such as the analysis of ribotypes (Vandenesch *et al.*, 1995), sequencing of the 16S rRNA gene (Spellerberg *et al.*, 1998), DNA–DNA hybridization (George & Kloos, 1994; Kawamura *et al.*, 1998) and the analysis of the banding patterns on gels of penicillin-binding proteins (Kanda *et al.*, 1991) have been used to detect *S. caprae* in various specimens from humans and to determine its involvement in human pathology. Seven previously documented cases of human infections with *S. caprae* have been reported (Elsner *et al.*, 1998; Spellerberg *et al.*, 1998; Vandenesch *et al.*, 1995): one case of endocarditis with macroscopic evidence of intraventricular vegetation, three cases of bacteraemia (one adult patient with catheter-associated sepsis and two neonates with cardiac malformations), two cases of symptomatic urinary tract infections in the absence of urethral catheter and one case of an intra-articular empyema following arthroscopic cruciate ligament repair in an adult immunocompetent patient.

Involvement of *S. caprae* in bone and joint infections

Frequent associations of *S. caprae* strains with bone and joint infections were reported by Shuttleworth *et al.* (1997) (10 of the 14 cases of human infections). In eight of the ten cases of bone and joint infections, the clinical significance of *S. caprae* was unclear because it was detected along with other pathogens in the same specimen. Moreover, its detection in several specimens from the same patient indicated that it was persistently present in the infected lesions and had a possible

predilection for bone sites. In our study, the four cases of human bone and joint infections following the implantation of orthopaedic material could be attributed to *S. caprae* strains detected in pure cultures in at least three specimens from the same patient. In each case, the orthopaedic material had to be removed 7–19 months after implantation. The recurrent infection of patient A (Table 1) was due to a single *S. caprae* strain with variations in the appearance of colonies, biochemical traits, slime production and drug-resistance patterns. The strain responsible for the recurrent infection was susceptible to the antibiotics used to treat the primary infection, so recurrence was not due to the selection of a resistant variant. For one of the four patients (patient C, Table 1), *S. caprae* infection of the knee prosthesis occurred one month after the end of antibiotic treatment for a primary infection caused by *S. epidermidis*. In this case too, the *S. caprae* strain responsible for the secondary infection was susceptible to the drugs used to treat the primary infection.

Adhesion factors and genes in *S. caprae*

Four of the ten human strains tested in this study produced slime, as did 33 % of 165 goat *S. caprae* strains including the type strain, CCM 3573^T, tested in a previous study (Bedidi-Madani *et al.*, 1998a). Phase variation in the expression of *in vitro* slime production has been reported in *S. epidermidis* strains (Baddour *et al.*, 1990; Christensen *et al.*, 1990; Deighton *et al.*, 1992; Ziebuhr *et al.*, 1997) and similar phase variation in *S. caprae* may account for the variable expression of this trait, in particular among the seven monoclonal strains of patient A. All the strains studied here, whether or not they produced slime, had nucleotide sequences that hybridized at low stringency (i) to the *S. epidermidis* *atlE* gene (Heilmann *et al.*, 1997; Rupp *et al.*, 1998) which encodes an autolysin binding vitronectin and responsible for the primary attachment to polystyrene, and for an increased virulence in a rat central venous catheter-associated infection model and (ii) to the *S. epidermidis* *ica* operon (Heilmann *et al.*, 1996) involved in the biosynthesis of an extracellular polysaccharide (PIA) (Mack *et al.*, 1996) and a capsular polysaccharide antigen called PS/A (McKenney *et al.*, 1998). Slime production may contribute to the virulence of *S. caprae* and to its protection against antibiotic access to the bacterium, as it does in *S. epidermidis* (Christensen *et*

al., 1983; Deighton *et al.*, 1996). The slime produced by some of the bacteria derived from the *S. caprae* strain of patient A may be why this strain was not eradicated by antibiotics. Alternatively, resistance to antibiotic treatment may be due to the segregation of a small-colony variant subpopulation of *S. caprae*. Such variants were shown to give *S. aureus* great flexibility favouring survival and making it difficult to cure infections in intra-vascular and bone sites despite the maintenance of antibiotic treatment over long time periods (Proctor & Peters, 1998).

There is increasing evidence that staphylococcal autolysins are important in pathogenesis (Hell *et al.*, 1998; Mani *et al.*, 1994; Rupp *et al.*, 1998). Three similar genes in *S. aureus*, *S. epidermidis* and *S. saprophyticus* encoding cell-wall-associated autolysins have been sequenced: *atl* (Oshida *et al.*, 1995, 1998), *atlE* (Heilmann *et al.*, 1997) and *aas* (Hell *et al.*, 1998), respectively. The three proteins encoded by these genes have two enzymic domains (amidase and glucosaminidase) in common. *S. epidermidis* AtlE binds to polystyrene and vitronectin whereas the *S. saprophyticus* Aas binds to fibronectin and sheep erythrocytes. The domain of Aas that binds to fibronectin and sheep erythrocytes is located between the two enzymic domains (Hell *et al.*, 1998). This prompted us to check whether the cell-wall fibronectin-binding proteins detected in all the *S. caprae* strains tested (Fig. 4) are encoded by the putative autolysin genes. N-terminal sequencing of the ~175 kDa protein of *S. caprae* strain 96007, which binds fibronectin, confirmed this assumption because the first ten amino acids were 80% identical to the corresponding sequence of the *S. aureus* Atl.

None of the *S. caprae* strains produced a clumping factor or showed any significant adhesion *in vitro* to fibrinogen but we cannot exclude the possibility that the genomes of all the strains contain several genes encoding fibrinogen-binding proteins, because several *HindIII* fragments were detected that hybridized at low stringency with *clfA*-2, encoding the serine-aspartate (SD) repeats of ClfA (Josefsson *et al.*, 1998; McDevitt *et al.*, 1994, 1995). If the hybridizing sequences are parts of genes encoding SD-repeat proteins, then it is a similar situation to that observed with the chromosome of *S. aureus* Newman strain hybridized with a probe for the SD-repeat region of *clfA* (Josefsson *et al.*, 1998). Two of these genes were *clfA* (McDevitt *et al.*, 1994) and *cflB* (Ni Eidhin *et al.*, 1998) which encode proteins binding the δ and α/β chains of fibrinogen, respectively, and the three other genes (*sdrC*, *sdrB*, *sdrE*) (Josefsson *et al.*, 1998) encode proteins suspected to interact with host proteins because of their organizational and sequence similarity with *clfA* and *clfB*.

Conclusions

This study enabled us to reveal, in addition to a gene encoding a new member of the serine-aspartate repeat protein family, *atlE* and *ica* genes in human and goat

strains of *S. caprae*. These latter genes are probably somewhat different from those used as probes because they were detected at low stringency only. All these genes should be isolated by cloning so that they can be characterized and their contribution to pathogenicity evaluated in animal models.

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