BACTERIAL PATHOGENICITY

Detection by PCR and analysis of the distribution of a fibronectin-binding protein gene (*fbn*) among staphylococcal isolates

T. MINHAS, H. A. LUDLAM, M. WILKS and S. TABAQCHALI*

Department of Medical Microbiology, St Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE

Summary. The fibronectin-binding proteins of *Staphylococcus aureus* are considered to be important virulence factors for colonisation and infection. The polymerase chain reaction (PCR) was used to detect part of a gene equivalent to the *fbnA* gene of *S. aureus* in 120 isolates of staphylococci (*S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. hominis*, *S. warneri*, *S. cohnii* and *S. lugdunensis*). Primers specific for the binding domain region of the *fbnA* gene of *S. aureus* produced PCR products of the predicted sizes (93 and 207 bp). The identity of the PCR products was confirmed by digestion with *DdeI* and nucleic acid hybridisation. The fibronectin-binding activity of the staphylococci was determined with a particle agglutination assay (PAA). The *fbn* gene was found to be present by PCR in 107 of the 120 staphylococci tested, irrespective of their site of isolation, and expression of the gene was detected by PAA in 101 of the 120 strains.

Introduction

Coagulase-negative staphylococci are an important and increasing cause of hospital-acquired infection. They are responsible for up to 26% of nosocomial bacteraemias, and are also the most common cause for the failure of prosthetic and implanted devices such as artificial heart valves, intravascular catheters, cerebrospinal fluid shunts and intraperitoneal catheters employed for continuous ambulatory peritoneal dialysis (CAPD).^{1–3}

Fibronectin plays a major role in adhesion between eukaryotic cells and has been shown to mediate adhesion between *Staphylococcus aureus* cells and host tissues. Fibronectin is a glycoprotein that is present in the body in two forms—a soluble dimeric form in fluids such as blood and plasma and a multimeric insoluble form deposited in extracellular matrix, connective tissue and implanted devices.⁴ Studies have shown that *S. aureus* synthesises fibronectin-binding proteins,⁵ and two highly homologous fibronectin-binding genes have been cloned and sequenced—*fbnA* and *fbnB*.^{6,7}

Świtalski *et al.*⁸ demonstrated fibronectin binding

among coagulase-positive and -negative staphylococci, but with considerable variation in binding activity both between and within species, leading to the suggestion that various growth parameters may explain the variation in binding. More recent studies have produced conflicting results with regard to the role of host serum proteins, including fibronectin, in mediating staphylococcal adherence.^{9,10} Furthermore, Valentin-Weigand *et al.*¹¹ demonstrated differences in adherence of coagulase-positive and -negative staphylococci *in vitro* depending on whether the fibronectin was in a soluble or immobilised form or incorporated into fibrin thrombi.

Since phenotypic expression of fibronectin-binding genes is variable, the aim of this study was to use the polymerase chain reaction (PCR) to investigate the distribution of a gene coding for a fibronectin-binding protein amongst staphylococci isolated either from sites of infection or as skin commensals. The primers were based upon nucleotide sequences common to both the *fbnA* and *fbnB* genes.

Materials and methods

Bacterial isolates

Thirty-eight staphylococcal isolates from the skin of healthy subjects, 30 isolates from patients with in-

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* Correspondence should be sent to Professor S. Tabaqchali.

Table. Distribution of fibronectin-binding genes and activity amongst staphylococcal isolates

Species (total number of isolates)	Skin isolates		CAPD peritonitis isolates		Endocarditis isolates	
	PCR + ve	PAA + ve	PCR + ve	PAA + ve	PCR + ve	PAA + ve
<i>S. aureus</i> (30)	11/11	10/11	9/9	8/9	9/10	8/10
<i>S. epidermidis</i> (33)	7/7	7/7	7/9	7/9	15/17	12/17
<i>S. haemolyticus</i> (16)	5/5	5/5	9/10	9/10	1/1	1/1
<i>S. simulans</i> (7)	5/6	6/6	—	—	1/1	1/1
<i>S. hominis</i> (6)	5/5	5/5	1/1	1/1	—	—
<i>S. warneri</i> (5)	2/4	2/4	1/1	1/1	—	—
<i>S. lugdunensis</i> (3)	—	—	2/2	1/2	1/1	1/1
<i>S. intermedius</i> (2)	—	—	1/2	1/2	—	—
<i>S. saprophyticus</i> (1)	—	—	1/1	1/1	—	—
<i>S. capitis</i> (2)	—	—	1/2	1/2	—	—

fective endocarditis and 37 isolates from CAPD-associated peritonitis were tested. The species distribution is shown in the table. In addition, the following reference strains were included: *S. aureus* NCTC 6571; *S. aureus* NCTC 08532; *S. auricularis* NCTC 12101; *S. capitis* NCTC 11045; *S. cohnii* NCTC 11041; *S. epidermidis* NCTC 11047; *S. haemolyticus* NCTC 11042; *S. hominis* NCTC 1320; *S. intermedius* NCTC 11048; *S. saprophyticus* NCTC 07292; *S. simulans* NCTC 11046; *S. warneri* NCTC 11044; *S. xylosus* NCTC 11043; *M. luteus* NCTC 02665; and *M. roseus* NCTC 07521. All isolates were collected and speciated as described by Ludlam *et al.*¹²

DNA preparation

Bacteria were cultured aerobically on blood agar (defibrinated horse blood 5% v/v in Oxoid Nutrient Broth no. 2 plus agar 1.5% w/v), harvested after incubation for 18 h at 37°C, and washed in phosphate-buffered saline (pH 7.3). The cells were then incubated in lysostaphin (Sigma) 0.2 mg/ml at 37°C for 3 h; the supernate was discarded after centrifugation at 3000 *g* for 2 min. The deposit was resuspended in 0.5 ml of 5 M guanidium thiocyanate (Sigma) and, after vortex mixing for 30 s, 0.25 ml of cold 7.5 M ammonium acetate was added. The solution was mixed by gentle inversion and left on ice for 10 min. Chloroform:2-pentanol (24:1, v:v) was added, mixed by inversion and vortex mixed for 20 s to form an emulsion. The emulsion was centrifuged at 13000 *g* for 5 min, 0.54 volumes of isopropanol were added to the upper layer, mixed and centrifuged as above to precipitate the DNA. The DNA was then washed three times with 1 ml of ethanol 70% v/v, dried under vacuum and dissolved in 100 µl of sterile water. The concentration of DNA was estimated by separation on agarose gels with 1-µg amounts of lambda-*Hind*III digests (Pharmacia) followed by visual comparison of the intensity of the bands.

Polymerase chain reactions

Primers were based on sequenced areas of the *fbnA* gene of *S. aureus* (the *fbnB* nucleotide sequence was almost identical in these regions) which were most likely to be highly conserved amongst staphylococci.⁶ The primers used were as follows: primer F1 (5'-GGTAATCAGTCATTCGAG-3'); primer R1 (5'-TGGCACACTGTCGAAGTC-3'); and primer Mr1 (5'-TGCGTAATAATGCTAAAC-3'). Primers were synthesised with a Model 391 EP Oligonucleotide Synthesiser (Applied Biosystems, Warrington, Cheshire).

The PCR mixture consisted of 70 µl of sterile distilled water, 16 µl of dNTPs (0.2 mM each; Pharmacia), 10 µl of reaction buffer (*Taq* polymerase buffer ×10; Amersham), 1 µl of each primer (20 nmol), 1 unit of *Taq* polymerase (Amersham) and 3 µl of template DNA (300 ng). The mixture was overlaid with 100 µl of sterile mineral oil. After an initial denaturation step (96°C for 2 min), 40 amplification cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 1 min were performed in a Hybaid Thermal Reactor, with a final 10-min extension step at 72°C. PCR products were separated on an agarose 1.5% w/v gel containing ethidium bromide (1 µg/ml) and visualised on an ultraviolet transilluminator.

Southern blotting and hybridisation procedure

The PCR-amplified products from various isolates were transferred to Hybond-N nylon membrane (Amersham). The 93-bp amplified product from *S. epidermidis* strain 1220.1 was used as the probe in hybridisation experiments. Hybridisations were carried out under conditions of high stringency (formamide 50% v/v, a hybridisation temperature of 42°C, and three washes performed at 42°C, 65°C and 42°C). Wash solution 1 contained 1 M Tris (pH 8.0), 3 M NaCl and 0.5 M EDTA; solution 2 was the same as solution 1, but also contained SDS 10% w/v; the third wash solution was a 10-fold dilution of the first wash solution. The probe was radiolabelled with [α -³²P]-

Fig. 1. Schematic illustration of part of the *fbnA* gene of *S. aureus* showing the sites to which primers F1, R1 and Mr1 annealed to produce amplified products. Repeating units are designated D1–D4.

dCTP (Amersham) by the random sequence hexamer method of Feinberg and Vogelstein.¹³ All blots were dried and exposed to Fuji-RX X-ray film at -70°C for 1–3 days.

Preparation of fibronectin-coated latex reagents

One ml of latex particle suspension (bead diameter $0.8\ \mu\text{m}$; Sigma) was mixed with 3 ml of $0.17\ \text{M}$ glycine–NaOH buffer (pH 8.2), centrifuged at $4500\ g$ for 5 min, and the pellet was resuspended in 3 ml of the same buffer. Highly purified fibronectin ($100\ \mu\text{g}$) from human plasma (Sigma) was added and mixed at 30°C for 12 h on a shaker. The mixture was centrifuged at $9200\ g$ for 5 min at room temperature and the supernate was discarded. The pellet was resuspended in 2 ml of the glycine–NaOH buffer containing ovalbumin (0.01% w/v; Sigma) and methiolate (0.01% w/v; Sigma), and kept at 4°C for 12 h.¹⁴

Particle agglutination assay (PAA)

Isolates were grown on blood agar at 37°C for 24 h. Cells were resuspended and washed in $0.02\ \text{M}$ potassium phosphate buffer (pH 6.8) and then resuspended in the same buffer to a density of MacFarland standard no. 5 ($c. 10^9$ cells/ml), followed by immediate testing in the PAA. Reactivity was observed by mixing one $20\text{-}\mu\text{l}$ drop of bacterial suspension on a glass slide with $20\ \mu\text{l}$ of fibronectin-coated latex particles. Any agglutination occurring within 2 min was recorded as positive, and any agglutination after 2 min was recorded as a negative result.¹⁴ The micrococci reference strains were included as negative controls and did not agglutinate. Uncoated beads were not used as negative controls because of the high incidence of non-specific agglutination of uncoated latex beads.¹⁵

Reproducibility

All PCR, PAA and hybridisation studies were performed in duplicate.

Results

Amplification of *fbn* gene fragments

Primers F1 and R1, based on the sequence data of the *fbnA* gene of *S. aureus*, were used in PCR reactions with template DNA from staphylococcal isolates. Fig. 1 illustrates the fibronectin-binding region of the gene to which the primers annealed to yield 93- and 207-bp products. The four repeating units (D1–D4) are adjacent to each other. Primer F1 annealed at two sites within the D1 and D2 units of the *fbn* gene, whereas primer R1 annealed to a single site within the D2 unit. Fig. 2 shows an example of the products obtained (93 and 207 bp) from 107 of the 120 strains with primers F1 and R1. The table shows the proportion of the 105 clinical isolates that were PCR-positive or -negative. The identity of the two PCR products was investigated by digesting the products with *DdeI* since a unique *DdeI* site exists within the D2 repeating unit of the *fbnA* gene of *S. aureus*. Fig. 3 shows the effect of digesting the 93- and 207-bp products with *DdeI*. A single band of $c. 40\text{--}50$ bp was observed when the 93-bp product was digested, instead of the two expected fragments of 52 bp and 41 bp, probably because of the limited resolution of the agarose gel. The larger PCR product (207 bp), which includes amplification of part of the adjacent D1 repeating unit in addition to the 93-bp product, was restricted at the same site and yielded two visible bands of 52 bp and 155 bp. As expected, the two strains of micrococci, *M. luteus* (NCTC strain 02665) and *M. roseus* (NCTC strain 07523), did not yield any amplification products. Not all isolates produced both a 93- and a 207-bp product.

The 93-bp amplified fragment from *S. epidermidis* strain 1220.1 was confirmed to be part of the *fbnA/fbnB* genes from nucleotide sequence data (data not shown). There was very close homology between the *S. aureus fbnA/B* gene sequence data and the nucleotide sequence of the PCR products.

Hybridisation of ^{32}P -PCR products to amplified products and digested DNA

Further confirmation of the identity of the PCR products was obtained by nucleic acid hybridisations.

Fig. 2. Example of PCR amplification of 207- and 93-bp products (with primers F1 and R1) visualised on an ethidium bromide-stained agarose 1.5% w/v gel. Lanes: a, *S. aureus* strain Abk; b, *S. aureus* Oxford strain; c, *S. aureus* strain Dhk; d, *S. epidermidis* strain DLe; e, Dhk negative control; f, *S. haemolyticus* strain Pts; g, *S. epidermidis* strain Ev; h, *S. hominis* strain Fst; i, *S. lugdunensis* strain Gly; j, *S. aureus* strain Wwd; k, *S. epidermidis* strain NCTC 11047; l, 100-bp ladder marker (Promega).

Fig. 3. Examples of the digestion of PCR products with *DdeI*. Lanes: a, *DdeI*-digested products of 155, 52 and 41 bp from *S. epidermidis* strain 1220.1; b, undigested PCR products of 207 and 93 bp from strain 1220.1; c, *DdeI*-digested products of 155 and 52 bp from *S. aureus* strain Dhk; d, undigested PCR product of 207 bp from strain Dhk; e, *DdeI*-digested products of 52 and 41 bp from *S. lugdunensis* strain Gly; and f, undigested PCR product of 93 bp from strain Gly.

Fig. 4. Autoradiograph of a Southern blot of the 207- and 93-bp PCR products from various staphylococcal species hybridised with the ^{32}P -labelled 93-bp amplified product from *S. epidermidis* strain 1220.1. Lanes: a, *S. epidermidis* strain 1220.1 (positive control); b, *S. hominis* strain Fst; c, *S. haemolyticus* strain Pst; d, *S. warneri* strain Mng; e, *S. epidermidis* strain Ev; f, *M. roseus* NCTC strain 07523 DNA (negative control); g, *S. lugdunensis* strain Gly; h, *S. simulans* strain Ly; i, *S. aureus* Oxford strain; j, *S. aureus* strain Dhk.

Fig. 4 shows the results of a high-stringency hybridisation reaction between amplified fragments from clinical staphylococcal isolates or skin commensals and the radiolabelled 93-bp PCR product from *S. epidermidis* strain 1220.1. This result indicated that the 93- and 207-bp PCR products were part of a gene equivalent to the *S. aureus fbnA* and *B* genes. The 207-bp product was also shown to include the 93-bp fragment, as indicated in fig. 1, because primer F1 anneals to two almost identical sites.

A 673-bp PCR product was amplified from *S. epidermidis* strain 1220.1 when primers F1 and Mr1 were used. The Mr1 primer was based on the sequence for the membrane-spanning region at the carboxyl terminal of the *fbn* gene. The 673-bp product was an extension of the 93- and 207-bp fragments (as confirmed by nested PCR; results not shown).

PAA

It is evident from the table that most (101 of 120) of the staphylococcal isolates, irrespective of their coagulase reaction or site of origin, agglutinated fibronectin-coated latex beads. However, one isolate from skin (*S. simulans*) repeatedly gave no PCR product, but a positive agglutination reaction. A comparison of the results obtained by PCR and PAA (table) indicated a good correlation between the two techniques. The PCR data showed a high level of distribution of the *fbn* gene among many species of staphylococci isolated from different sites. The DNA of 95 clinical isolates produced either 93- or 207-bp, or both, PCR products. Most of this "positive group" (92 of 95) had the 93-bp product, although many of them also had the 207-bp fragment (74 of 95). A few isolates (3 of 95) produced only the 207-bp product following PCR amplification.

Discussion

The increasing problem of nosocomial infection with coagulase-negative staphylococci has provided an urgent stimulus to investigate the poorly understood pathogenic mechanisms and virulence determinants of these opportunist pathogens. Increasing evidence of the importance of fibronectin-binding proteins in colonisation by *S. aureus* has stimulated an interest in the interaction of coagulase-negative staphylococci with fibronectin. To date there have been few studies of this interaction, all of which have been at the phenotypic level, with variable results attributed to different laboratory methodology.^{11, 16} This study attempted, firstly, to shed more light on the accuracy of tests for fibronectin binding by focusing on the development of a rapid and sensitive method for detecting the fibronectin-binding gene among staphylococci, and secondly, to assess the pathogenic importance of fibronectin binding by comparing skin commensals and clinical isolates.

Primers based on the *fbnA* gene of *S. aureus* were used to detect this or an equivalent gene among coagulase-negative staphylococci. The primers (F1 and R1) based on a repeating unit region (D repeats) produced amplified products consistently from 107 of

the 120 isolates tested. It is likely that this area of the gene is highly conserved, as most species of staphylococci tested produced either 93- or 207-bp (or both) products; this was confirmed for *S. epidermidis* strain 1220.1 by nucleotide sequence data (unpublished results). The most likely explanation for both products not being amplified in all of the 107 isolates is that of genetic polymorphism at the annealing sites. In the isolates where a product was amplified, it was assumed that the whole *fbn* gene was present, and this was confirmed for 106 of the 107 isolates by demonstrating activity by PAA. The PAA^{14, 15} was chosen as a rapid screening method primarily because of the technical disadvantages of using the ¹²⁵I-fibronectin technique,⁵ and the PAA method proved to be reliable and reproducible under the in-vitro test conditions described.

The strong correlation between the PAA data and the PCR results confirmed that the whole *fbn* gene was present in most isolates. Seven of the 120 isolates produced a PCR product, but did not agglutinate fibronectin-coated latex particles. Possibly the cells could bind fibronectin in its immobilised form. Valentin-Weigand *et al.*¹¹ have demonstrated varied ability among staphylococci to bind to different forms of fibronectin (i.e., soluble, immobilised and incorporated).

One skin isolate of *S. simulans* produced no PCR product, but the cells agglutinated fibronectin-coated latex particles. This could possibly result from either autoagglutination by the cells (a false PAA-positive) or a false-negative by the PCR reaction. The latter may be possible if the D-repeating region of this isolate has low homology with the *S. aureus fbnA* gene or if there is polymorphism at the primer annealing site(s).

Overall, this study has detected the presence of the *fbn* gene (or its equivalent) in 90% of coagulase-positive and -negative staphylococci, irrespective of whether the isolates were regarded as clinically significant or commensals on the skin. The fact that this gene is present and being expressed in such a high proportion of coagulase-negative staphylococci suggests that fibronectin production *per se* is unlikely to be an important virulence determinant.

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