A low-fibronectin-binding mutant of *Staphylococcus aureus* 879R4S has Tn918 inserted into its single fnb gene

C. Greene,† P. E. Vaudaux,² P. Francois,² R. A Proctor,³ D. McDevitt¹† and T. J. Foster¹

INTRODUCTION

The ability of pathogens to attach to host cells or tissues is an important step in the initiation of infection. *Staphylococcus aureus* can adhere to host matrix proteins such as fibrinogen, collagen, laminin and vitronectin (Boden & Flock, 1989; Chhatwal et al., 1987; Holderbaum et al., 1987; Lopes et al., 1985; McDevitt et al., 1994; Speziale et al., 1986). The organism also binds to fibronectin, both in solution and on surfaces (Bozzini et al., 1992). The fibronectin-binding activity has been shown to be due to high-molecular-mass cell-wall-associated proteins (fibronectin-binding proteins, FnBPs). Strain 8325-4 expresses two distinct but related FnBPs called FnBPA and FnBPB (Jonsson et al., 1991; Signas et al., 1989). These are encoded by closely linked but separately transcribed fnb genes (Greene et al., 1995) (Fig. 1), both of which must be inactivated to eliminate bacterial interactions with fibronectin.

The FnBPs are composed of distinct domains. Region A is the most divergent (approximately 45% residue identity). The B region of FnBPA (repeats of 30 residues) is lacking altogether in FnBPB. In contrast, regions C, D, W and M are very similar. Regions W and M are required for sorting of the FnBPs to the surface and for attachment to the cell wall (Navarre & Schneewind, 1994; Schneewind et al., 1993). The ligand-binding activity is located in region D (Raja et al., 1990; Signas et al., 1989) which is composed of three repeats of 38 residues (D1–D3) plus one incomplete repeat. Each D repeat can bind fibronectin. The minimum fibronectin-binding region occurs within residues 15–36 (McGavin et al., 1991).

FnBPs have been isolated from a number of streptococcal species (Hanks & Caparon, 1992; Lindgren et al., 1992; Speziale et al., 1986). Comparison of the amino acid sequences of the fibronectin-binding domains of staphylococcal and streptococcal FnBPs has identified a core fibronectin-binding sequence EDT/S–(X9,10)–GG–(X3,4)–I/VDF (McGavin et al., 1993).

A low-fibronectin-binding mutant of strain 879R4S was isolated by Kuyper & Proctor (1989) but the insertion site was not characterized. The mutant, *S. aureus* 879R4S/l536, was shown to be significantly less virulent in a rat model for...
endocarditis suggesting that attachment to fibronectin is an important step in the pathogenesis of this disease.

In this study the structure of the fnb locus of strain 879R4SSp was analysed and compared to that of strain 8325-4. The insertion site of Tn918 was mapped by Southern hybridization and identified precisely by sequencing a PCR-amplified junction fragment between Tn918 and the fnb gene.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. Strain 879R4S and the Tn918 mutant 879R4S/1536 were used for adherence and protein analysis while the spectinomycin-resistant derivatives 879R4SSp and 879R4SSp/1536 were used for DNA analysis.

Bacterial growth. S. aureus strains were grown routinely in trypticase soy broth or agar, and Mueller–Hinton broth was used to grow cultures for adherence assays and protein analysis. *Escherichia coli* strains harbouring plasmids were grown in LB broth and L-agar (Miller, 1972) containing ampicillin at 100 μg ml⁻¹.

Manipulation of DNA. DNA-modifying enzymes were purchased from New England Biolabs and were used according to the manufacturer’s instructions. DNA manipulations were performed using standard procedures (Ausubel et al., 1987; Sambrook et al., 1989). DNA hybridization was performed by the method of Southern (1975). S. aureus genomic DNA was purified by the method of Lindberg et al., (1972). Probe DNA was labelled with [α-³²P]dATP by the random primer method using the Prime-A-Gene kit (Promega). Probe A was a 1.2 kb fragment corresponding to bases 147–1344 of the fnbA gene of strain 8325-4. It was isolated from plasmid pFR040 by cleavage with EcoRI and XbaI. Probe B corresponded to bases 113–1635 of the fnbB gene of 8325-4. This fragment was released from plasmid pFR060 with AccI. One AccI site is located within the multiple cloning site of pUC18 and the other in the fnbB gene. These probes comprise sequences that are specific for fnbA and fnbB, respectively. Probe C was isolated from pFR001 by cutting with SpeI (site in pBR322) and EcoRI (site in fnbA gene). It carries 1 kb of DNA 5’ to fnbA and the first 147 bp of the open reading frame. Sequencing was carried out with fluorescently labelled SP6 and T7 primers using an Applied Biosystems automated sequencer.

Primers that specifically amplified fnbA (330–1674 bp) and fnbB (328–1489 bp) by recognizing unique sequences in region A of each gene were described by Greene et al. (1995).

Amplification of a Tn918–fnb junction fragment. A Tn918–fnb junction fragment from S. aureus 879R4SSp/1536 was amplified from chromosomal DNA using Vent DNA polymerase and the oligonucleotides 5’-GACGGTACCTGAGTGGTTTTGACC-3’ (forward 3) or 5’-GCAGGTACCATGACGCTGAACTAT-3’ (forward 4), corresponding to the right-hand and left-hand ends of Tn916, respectively (Clewell et al., 1988) and 5’-GGTGGTACCTGTTGCGTTATATGATTG-3’ (reverse 3) corresponding to bases 219–238 of the fnbA gene of S. aureus 8325-4. All primers had a 9 bp extension with a KpnI site (underlined) incorporated into their 5’ ends. PCR reaction

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* Ap', ampicillin resistance.
Fig. 1. Southern hybridization analysis of *S. aureus* strains 8325-4 and 879R4Sp. (a, b) Chromosomal DNA of strains 8325-4 (lanes 1, 3 and 5) and 879R4Sp (lanes 2, 4 and 6) was cut with Accl (lanes 1 and 2), Clal (lanes 3 and 4) or PvuII (lanes 5 and 6), electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane and hybridized with (a) a 1.2 kb probe specific for the fnbA gene of 8325-4 or (b) a 1.5 kb probe specific for the fnbB gene of 8325-4. (c) Map of the fnb locus of 8325-4 and probes A and B. Regions marked S, A, B, C, D, W and M are domains in the FnBPA and FnBPB proteins. The 3056 bp fnbA gene is separated from the 2823 bp fnbB gene by a 682 bp intragenic region.

mixtures contained 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris/HCl (pH 8.8), 2 mM MgSO₄, 0.1% Triton X-100, 10 ng template DNA (*S. aureus* 879R4SSp/1536 chromosomal DNA), 2 mM of each dNTP, 200 mM forward and reverse primers, and 2 units Vent DNA polymerase in a 100 ml volume. Cycling parameters began with an initial denaturing step of 94 °C for 7 min, followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min, and ended with a 10 min extension step at 72 °C.

Adhesion to fibronectin-coated coverslips. Purified human fibronectin (Chemicon) was dissolved in PBS [0.145 M NaCl, 0.15 M sodium phosphate (pH 7.3); oxoid] at 1 mg ml⁻¹ and stored at −70 °C. The concentration was measured spectrophotometrically using A₂₈₀ 1.28. The attachment properties of *S. aureus* strains were measured using a previously described adhesion assay with poly(methyl methacrylate) (PMMA) coverslips coated in vitro with purified fibronectin (Vaudaux et al., 1984, 1995). To optimize absorption of fibronectin from concentrations below 1 µg ml⁻¹, the PMMA coverslips were precoated with gelatin (1 mg ml⁻¹). After rinsing in PBS, they were incubated for 60 min at 37 °C with low concentrations (ranging from 0.125 to 1 µg ml⁻¹) of fibronectin followed by rinsing in PBS. PMMA surfaces were precoated in a dose-dependent manner with fibronectin ranging from 24 to 92 ng per coverslip (Vaudaux et al., 1993). The adhesion characteristics of *S. aureus* strains were evaluated by incubating coverslips with 4 × 10⁶ c.f.u. washed exponential-phase cells, radiolabelled with [³H]thymidine during growth in Mueller–Hinton broth.

SDS-PAGE and Western affinity blotting. Bacteria were grown to stationary phase without shaking in Mueller–Hinton broth at 37 °C. Cells were harvested by centrifugation at 5000 g and washed twice in PBS. Bacteria were lysed in 1.5 ml PBS containing lysozyme (AMBI; 20 µg ml⁻¹), DNase (10 µg ml⁻¹), aprotinin (78 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 mM), pepstatin A (0.7 mM), phenylmethanesulfonyl fluoride (0.25 mM) and dithiothreitol (1 mM) (all from Sigma) for 15 min at 37 °C. The lysate was centrifuged at 5000 g for 10 min and the supernatant retained. The protein concentration was determined by the BCA method (Pierce) and adjusted to 3 mg ml⁻¹ with PBS. Aliquots were boiled in Laemmli buffer (Laemmli, 1970) and separated by SDS-PAGE using 6% (w/v) acrylamide. Proteins were transferred to an Immobilon membrane (Millipore) using a liquid transblot system (BioRad). The membrane was blocked in 10 mM Tris/HCl (pH 8.0), 500 mM NaCl, 0.1% Tween 20 (Fluka) (TBST) containing 5% (w/v) skimmed milk and incubated with pure human fibronectin (30 µg ml⁻¹ in TBST). The membrane was rinsed several times in TBST and incubated...
with monoclonal antibody mAb-1936 raised against the N-terminus of fibronectin (1:5000, Chemicon) followed by peroxidase-conjugated anti-mouse IgG (1:5000; Amersham). Detection was by enhanced chemiluminescence (Amersham).

RESULTS

The fnb locus of S. aureus 879R4Sp

S. aureus strains 8325-4 and Newman have two closely linked chromosomal genes, fnbA and fnbB, encoding fibronectin-binding proteins (Jonsson et al., 1991; Signas et al., 1989). To investigate the structure of the fnb locus of strain 879R4Sp, genomic DNA of this strain and strain 8325-4 was cleaved with restriction enzymes AciI, Clal and PstI and analysed by Southern hybridization.

Fig. 1(a) shows blots probed with labelled DNA specific for the fnbA gene of S. aureus 8325-4 (bases 147–1344 spanning region A) while Fig. 1(b) shows the same DNA probed with a fragment specific for fnbB (bases 113–1635). With the fnbA probe signals of similar intensity occurred in each lane suggesting that 879R4Sp has an fnb gene which is related to fnbA of 8325-4. In contrast, the fnbB-specific probe hybridized only weakly to 879R4Sp DNA. Furthermore, the weakly hybridizing bands were of the same size as those which hybridized to the fnbA probe whereas for each enzyme a different sized fragment reacted in the 8325-4 samples. Also, in each sample of 8325-4 DNA probed with the fnbA-specific probe a second faint band which was the same size as the more intense band in the corresponding reaction with the fnbB-specific probe was seen. Similarly, a faint band occurred in the fnbB-probed samples which corresponded in size to the major band in the fnbA reactions. This is due to slight cross-reaction occurring between the fnbA probe and the fnbB gene and vice versa. Second fainter bands were not seen with 879R4Sp DNA and the fnbA probe. These data strongly suggest that 879R4Sp has a single fnb gene which is closely related to fnbA of 8325-4.

Further restriction mapping experiments (not shown) revealed some conserved restriction sites (PstI and EcoRI) within the fnbA genes of 879R4Sp and 8325-4. However, several polymorphic sites including Clal sites were noted (see Fig. 3).

Genomic DNA of strain 879R4Sp was amplified by PCR with primers that specifically recognize unique sequences in region A of fnbA and fnbB of strain 8325-4 (Greene et al., 1995). The fnbA-specific primers gave a fragment of 1.3 kb in both 8325-4 and 879R4Sp (Fig. 2, lanes 1 and 3). In contrast the fnbB-specific primers amplified a fragment of about 1 kb in 8325-4 but did not amplify a fragment from 879R4Sp (Fig. 2, lanes 2 and 4), which also suggests that the fnbB gene is absent in this strain.

Analysis of the Tn918 insertion in strain 879R4Sp/1536

Kuyipers & Proctor (1989) showed that there is a single Tn918 insertion in the low-fibronectin-binding strain 879R4Sp/1536. They transduced the Tn918 insertion from the parental strain 879R4S to the spectinomycin-resistant derivative 879R4Sp and showed by Southern hybridization that the transposon was located in the same sized EcoRI fragment, indicating that Tn918 was acquired by homologous recombination. To determine if the 16.8 kb transposon Tn918 (Clewell et al., 1985) is located in the fnbA gene, genomic DNA of strains 879R4Sp and 879R4Sp/1536 was analysed by Southern hybridization. DNA was cleaved with EcoRI, HindIII, ClaI and PstI and hybridized with probe A from the fnbA gene of 8325-4. For EcoRI, ClaI and PstI there were no differences in the sizes of the hybridizing fragments between the wild-type and the mutant (data not shown). However, a large (>15 kb) HindIII fragment in 879R4Sp DNA was about 2 kb larger in the mutant. As Tn918 carries a HindIII site, this must be the right junction fragment of Tn918 and the >15 kb chromosomal HindIII fragment. It should also be noted that the single ClaI site in the 879R4Sp fnbA gene is quite close to the 5' end of the gene and is either very close to the end of the region covered by probe A, or outside it. This explains why the ClaI fragment hybridizing with probe A did not change in the mutant.

DNA was then analysed with probe C corresponding to 5' non-coding DNA and the first 147 bp of fnbA. The hybridization pattern of the parent and mutant samples differed for each enzyme tested (Fig. 3). For EcoRI and ClaI the 879R4Sp fragments of 5.5 kb and 4 kb, respectively, had increased to >15 kb, presumably due to the insertion of Tn918. In the case of HindIII, two bands hybridized to the probe in the 879R4Sp/1536 sample. The larger corresponds to the single larger fragment seen.
**Fig. 3.** Southern hybridization analysis of the insertion site of Tn918 in *S. aureus* strain 879R4SSp/1536. (a) Chromosomal DNA of 879R4SSp (lanes 1, 3, 5 and 7) and 879R4SSp/1536 (lanes 2, 4, 6 and 8) was cut with EcoRI (lanes 1 and 2), HindIII (lanes 3 and 4), Clal (lanes 5 and 6) or PstI (lanes 7 and 8), electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane and hybridized with probe C, a 1.1 kb fragment corresponding to the 5' flanking region and the first 147 bp of fnbA of *S. aureus* 8325-4. (b) Map of the predicted structure of the fnb locus of strain 879R4SSp/1536. The PCR primers used to amplify the Tn918-fnb junction fragment are indicated. Restriction sites deduced from mapping experiments are shown in parentheses. C, Clal; P, PstI, E, EcoRI; H, HindIII.

with probe A. Tn918 has a single HindIII site and probe C must hybridize to DNA flanking both the left-hand and right-hand ends of the transposon. (Tn918 does not have EcoRI, Clal or PstI sites so only a single larger fragment hybridized in the mutant samples.) A PstI fragment of 879R4SSp of about 15 kb was also significantly larger in the mutant, but it was not possible to measure fragment sizes accurately under the conditions used. It was concluded that Tn918 had probably inserted into non-coding DNA 5' to the fnbA gene or within the open reading frame very close to the 5' end.

**Mapping the Tn918 insertion site by PCR and DNA sequencing**

To map precisely the site of the Tn918 insertion in the fnbA locus of 879R4SSp/1536, a junction fragment between Tn918 and the fnbA gene was amplified by PCR.
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The fnbA gene showed that Tn918 was located between bases 8325-4 in the 41 bp of 5' non-coding sequence and the Tn978 sequence. Plain type denotes differences between the 879R4SSp and the 8325-4 fnbA gene. Bold type denotes the Tn918 sequence. Plain type denotes 5' non-coding sequence.

Fig. 4. Sequence of the Tn918-fnb junction fragment of S. aureus strain 879R4SSp/1536. A fragment carrying the junction of Tn918 and the fnbA gene of 879R4SSp/1536 was amplified by PCR, cloned into pGEM-7Zf(+) and sequenced. Bold type denotes Tn918 sequence. Plain type denotes differences in the FnBPA amino acid sequences.

Since the orientation of Tn918 was not known, PCR forward primers were designed to coincide with DNA sequence 74 bp inside either end of the transposon. A single reverse primer corresponded to bases 219-238 of the 8325-4 fnbA structural gene (Fig. 3). The fragment was sequenced and comparison with the 8325-4 fnbA gene showed that Tn918 was located between bases -41 and -42 5' to the fnbA structural gene (Fig. 4). Although the fnbA coding sequence was not disrupted by the transposon, expression of the gene is likely to be affected because transcription from the fnbA promoter, located at bases -85 to -57 in strain 8325-4 (Greene et al., 1995) would be prevented. A fragment of the same size was amplified from the spectinomycin-sensitive strain 879R4SS/1536 (data not shown) which demonstrates that the transposon is located at the same site in both strains.

The fnbA gene of strain 879R4SSp was 96.8% identical to fnbA of 8325-4 in the 41 bp of 5' non-coding sequence and in the coding sequences between bases 1 and 237. There were only nine mismatches over a stretch of 278 bp. Eight base differences occurred in the coding sequence; two were silent while the other six caused five amino acid substitutions (Fig. 4).

Expression of fibronectin-binding proteins

Western ligand affinity blotting was performed on proteins released from the cell walls of strains 879R4S and the mutant 879R4S/1536 (Fig. 5). A single reactive protein of about 180 kDa was observed. The native form of FnBPA of 8325-4 had a predicted molecular mass of 108 kDa but was observed to migrate in SDS-PAGE at about 200 kDa (Greene et al., 1995). The intensity of the 180 kDa protein in the 879R4S/1536 sample was greatly reduced, which is consistent with the Tn918 insertion being located 5' to, and not within, the fnbA gene. A promoter-like sequence was identified in the left-hand end of the closely related transposon Tn916 (Clewell et al., 1988). This sequence is conserved in Tn918 (Fig. 4) and may allow a low level of expression of FnBPA in the mutant. The intense band of about 50 kDa was protein A which bound the peroxidase-conjugated anti-fibronectin antibody. Similar results were obtained with the spectinomycin-resistant derivatives 879R4SSp and 879R4SSp/1436 (data not shown).

Defective attachment of the low-fibronectin-binding mutant to surface-bound fibronectin

Adhesion of the parental strain S. aureus 879R4S was linearly promoted by low amounts (< 50 ng per coverslip) of fibronectin immobilized on PMMA surfaces, then increased marginally at higher amounts of immobilized fibronectin (Fig. 6). At all concentrations of fibronectin, the low-fibronectin-binding mutant 879R4S/1536 showed a markedly decreased, but still significant, attachment compared to the parental strain. In contrast the double fnbA fnbB mutant of 8325-4 was completely defective (Greene et al., 1995). Similar results were obtained with the spectinomycin-resistant derivatives 879R4SSp and 879R4SSp/1436 (data not shown).

DISCUSSION

S. aureus strains 8325-4 and Newman express two FnBPs encoded by separately transcribed fnb genes. Data presented here suggest that S. aureus 879R4S has only one gene which is closely related to fnbA of strain 8325-4.
The low-fibronectin-binding mutant 879R4SSp/1536 has a copy of transposon Tn918 inserted between the promoter and coding sequence of its fnbA gene. The fnbA gene of strain 879R4S is almost 97% identical to fnbA of 8325-4 in the 237 bp of the coding sequence and the 41 bp of 5' non-coding sequence. Conserved PstI and EcoRI sites are located in this region. Given the similarity of coding and 5' non-coding sequences, the 879R4S fnbA promoter is likely to be the same as that of 8325-4 fnbA.

The promoter was mapped by primer extension between bases −85 and −57 (Greene et al., 1995). The insertion site of Tn918 is between bases −41 and −42 and is thus situated between the promoter and coding sequence of the fnbA gene. This is consistent with reduced adherence to fibronectin-coated PMMA coverslips and to the reduced levels of FnBPA detected by Western ligand affinity blotting. A potential promoter is located at the right-hand end of Tn918 (Clewel et al., 1988) (Fig. 4). This is not likely to be a strong promoter as the spacing between the −35 and −10 regions is 18 bp, which is not optimal for high-level expression, and there are several differences from the consensus E. coli promoter sequence (Hawley & McClure, 1983). Nevertheless, it may be active enough to enable low-level transcription of the fnbA gene of 879R4S/1536. This might explain the low-level expression of the FnBP and the reduced, but still significant, adherence of the mutant.

The use of transposon mutagenesis to make fibronectin-binding-defective mutants was successful in the case of S. aureus 879R4S because there is only one fnb gene. This approach is unlikely to have worked with S. aureus 8325-4 because this strain has two fnb genes which are transcribed independently. The two fnb genes encode distinct FnBPs and both must be inactivated to abolish the ability of 8325-4 to bind to fibronectin-coated surfaces (Greene et al., 1995). Tn918 mutagenesis would not abrogate expression of two fnb genes unless the insertion caused an adjacent deletion to inactivate both genes.

Many studies have shown that S. aureus binds to fibronectin in vitro (Maxe et al., 1987; Raja et al., 1990; Greene et al., 1995). However, it is not clear if this is an important factor in the initiation of staphylococcal infections. Fibronectin is a component of the many host proteins deposited on the surface of implanted biomaterials. Adherence assays using biomaterials explanted from human patients showed fibronectin to be active in promoting attachment of S. aureus ((Vaudaux et al., 1989, 1993). Also, a mutant of S. aureus 8325-4 defective in fibronectin-binding did not bind to PMMA coverslips that had been removed from a subcutaneously implanted tissue cage implying that a significant quantity of fibronectin had been deposited in vivo (Greene et al., 1995). This suggests that fibronectin is an important component of the host proteins coating an implant and may be responsible for promoting bacterial attachment in vivo.

A rat endocarditis model was used by Kuyers & Proctor (1989) to examine the role of fibronectin in the initiation of infection. Cardiac endothelial trauma was produced in rats by inserting a catheter into the left ventricle (Santoro & Levison, 1978). Animals were challenged with S. aureus 879R4SSp or the low-fibronectin-binding mutant 879R4SSp/1536. Colonization of the left side of the heart of rats challenged with strain 879R4SSp/1536 was 250-fold lower than in rats challenged with the wild-type strain.

A mutant of S. aureus Newman defective in the fibrinogen-binding protein clumping factor also exhibited reduced virulence in the rat endocarditis model (Moreillon et al., 1995). This suggests that adherence to cardiac vegetations is multifactorial. We are currently testing this by measuring the virulence of a null mutant of 8325-4 lacking fnbA and fnbB, and a mutant lacking both fibronectin- and fibrinogen-binding proteins.

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**REFERENCES**


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