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

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A low-fibronectin-binding mutant of *Staphylococcus aureus* strain 879R4SSp generated by transposon Tn918 mutagenesis is attenuated in a rat endocarditis model (J. M. Kuypers & R. A. Proctor, 1989, *Infect Immun* 57, 2306–2312). PCR and Southern hybridization analysis with primers and probes, respectively, for the *fnbA* and *fnbB* genes of strain 8325-4 showed that strain 879R4SSp possesses a single *fnb* gene which is homologous to *fnbA*. This was confirmed by sequencing 41 bp of 5' non-coding and 237 bp of 5' coding DNA, which showed 97% base identity to *fnbA*. Southern hybridization and sequencing showed that Tn918 was inserted 41 bp 5' to *fnbA* in the mutant 879R4SSp/1536, between the promoter and initiation codon. Reduced adherence of the mutant to surface-bound fibronectin correlated with lower expression of a 180 kDa wall-associated fibronectin-binding protein.

Keywords: *Staphylococcus aureus*, fibronectin-binding protein, endocarditis, adherence, virulence

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 (Hanski & 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 879R4SSp caused by insertion of transposon Tn918 was isolated by Kuypers & Proctor (1989) but the insertion site was not characterized. The mutant, *S. aureus* 879R4SSp/1536, was shown to be significantly less virulent in a rat model for

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Abbreviations: FnBP, fibronectin-binding protein; PMMA, poly(methyl methacrylate).

The EMBL accession number for the *fnbA* sequence reported in this paper is X95848.

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 L-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 *SphI* (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'-GACGGTACCTGAGTTGGTTTGGACC-3' (forward 3) or 5'-GCAGGTACCATGACGCTGAACAT-3' (forward 4), corresponding to the right-hand and left-hand ends of Tn918, respectively (Clewett *et al.*, 1988) and 5'-CGTGGTACCTGTTGCGTTATATGATTG-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

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or [marker*]	Relevant properties	Source or reference
Strains			
<i>E. coli</i>			
TB1	<i>ara thi rpsL bsdR</i> ($r_k^- m_k^+$) $\Delta(lac-proAB)$ [$\phi 80d lac\Delta(lacZ)$ M15]		Yanisch-Perron <i>et al.</i> (1985)
<i>S. aureus</i>			
879R4S	<i>fnbA</i> ⁺	Restriction negative	Kuypers & Proctor (1989)
879R4SSp	<i>fnbA</i> ⁺	Restriction negative, spectinomycin resistant	Kuypers & Proctor (1989)
879R4S/1536	<i>fnbA::Tn918</i>	Tn918 mutant of 879R4S defective in fibronectin binding	Kuypers & Proctor (1989)
879R4SSp/1536	<i>fnbA::Tn918</i>	Tn918 mutant of 879R4SSp defective in fibronectin binding.	Kuypers & Proctor (1989)
8325-4	<i>fnbA</i> ⁺ <i>fnbB</i> ⁺	Transduced from 879R4S/1536 8325 cured of prophages	Novick (1963)
Plasmids			
pFR001	Ap ^r	<i>fnbA</i> ⁺ <i>fnbB</i> lacking 1.1 kb of 3' sequence	Signas <i>et al.</i> (1989)
pFR040	Ap ^r	3.5 kb insert of <i>fnbA</i> lacking 147 bp of 5' sequence	Jonsson <i>et al.</i> (1991)
pFR060	Ap ^r	3.2 kb insert of <i>fnbB</i> lacking 113 bp of 5' sequence	Jonsson <i>et al.</i> (1991)
pGEM-7Zf(+)	Ap ^r	Cloning vector	Promega

* Ap^r, ampicillin resistance.

Fig. 1. Southern hybridization analysis of *S. aureus* strains 8325-4 and 879R45Sp. (a,b) Chromosomal DNA of strains 8325-4 (lanes 1, 3 and 5) and 879R45Sp (lanes 2, 4 and 6) was cut with *AccI* (lanes 1 and 2), *ClaI* (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 $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl (pH 8.8), 2 mM MgSO_4 , 0.1% Triton X-100, 10 ng template DNA (*S. aureus* 879R45Sp/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 A_{280} 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 (Vaudoix *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 shown to be coated in a dose-dependent manner with fibronectin ranging from 24 to 92 ng per coverslip (Vaudoix *et al.*, 1993). The adhesion

characteristics of *S. aureus* strains were evaluated by incubating coverslips with 4×10^6 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 lysostaphin (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* 879R4SSp

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 879R4SSp, genomic DNA of this strain and strain 8325-4 was cleaved with restriction enzymes *AccI*, *ClaI* and *PvuII* 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 879R4SSp has an *fnb* gene which is related to *fnbA* of 8325-4. In contrast, the *fnbB*-specific probe hybridized only weakly to 879R4SSp 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 879R4SSp DNA and the *fnbA* probe. These data strongly suggest that 879R4SSp 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 879R4SSp and 8325-4. However, several polymorphic sites including *ClaI* sites were noted (see Fig. 3).

Genomic DNA of strain 879R4SSp 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 879R4SSp (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 879R4SSp (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 879R4SSp/1536

Kuypers & Proctor (1989) showed that there is a single Tn918 insertion in the low-fibronectin-binding strain 879R4SSp/1536. They transduced the Tn918 insertion

Fig. 2. PCR analysis with *fnbA*- and *fnbB*-specific primers. (a) Genomic DNA from *S. aureus* strains 8325-4 (lanes 1 and 2) and 879R4SSp (lanes 3 and 4) was amplified with *fnbA*-specific (lanes 1 and 3) or *fnbB*-specific (lanes 2 and 4) primers. (b) Map showing positions of the *fnbA*- and *fnbB*-specific primers in the 8325-4 *fnb* locus.

from the parental strain 879R4S to the spectinomycin-resistant derivative 879R4SSp 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 (Clewett *et al.*, 1985) is located in the *fnbA* gene, genomic DNA of strains 879R4SSp and 879R4SSp/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 879R4SSp 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 879R4SSp *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 879R4SSp 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 879R4SSp/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 *Eco*RI (lanes 1 and 2), *Hind*III (lanes 3 and 4), *Cla*I (lanes 5 and 6) or *Pst*I (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, *Cla*I; P, *Pst*I; E, *Eco*RI; H, *Hind*III.

with probe A. Tn918 has a single *Hind*III site and probe C must hybridize to DNA flanking both the left-hand and right-hand ends of the transposon. (Tn918 does not have *Eco*RI, *Cla*I or *Pst*I sites so only a single larger fragment hybridized in the mutant samples.) A *Pst*I 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.

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 *fnbA* sequence. Differences between the 879R4SSp and 8325-4 *fnbA* genes are shown, as well as changes in the FnBPA amino acid sequences.

Since the orientation of Tn918 was not known, PCR forward primers were designed to coincide with DNA 74 bp inside either end of the transposon. A single reverse primer corresponded to bases 219–238 of the 8325-4 *fnbA* gene (Fig. 3).

A product of 370 bp was amplified by primers F3 and R3, while primers F4 and R3 did not yield a product. 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 879R4S/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

Fig. 5. Western ligand affinity blotting analysis of proteins extracted by lysostaphin treatment from the cell wall of *S. aureus* strains 879R4S (lane 1) and 879R4S/1536 (lane 2).

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 (Clewett *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 879RS/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.

Fig. 6. *In vitro* adhesion of *S. aureus* strain 879R4S (○) and the low-fibronectin-binding mutant 879R4S/1536 (●) to gelatin-PMMA coverslips coated with human fibronectin. Mean values are shown $\pm$ SEM ($n = 3$).

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 *Pst*I and *Eco*RI 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 (Clewett *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 Kuypers & 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.

ACKNOWLEDGEMENTS

This work was supported by the Health Research Board of Ireland, by The Wellcome Trust (project grant 033403), by the Swiss National Foundation (grants 32-30161 and 5002-35180), by Ohmeda (Swindon), by the US National Institute of Health (HL-29586) and by the Graduate and Medical School Department of Medicine, University of Wisconsin. We thank Manuela Bento for technical assistance.

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Received 10 November 1995; revised 8 March 1996; accepted 13 March 1996.