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Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes

C. Greene,[1] D. McDevitt,[1†] P. Francois,[2] P. E. Vaudaux,[2] D. P. Lew[2] and T. J. Foster[1*]
[1]*Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland.*
[2]*Division of Infectious Diseases, Hôpital Cantonal Universitaire, 1211 Geneva 4, Switzerland.*

Summary

***Staphylococcus aureus* 8325-4 has the potential to express two distinct cell wall-associated fibronectin-binding proteins called FnBPA and FnBPB. In order to test if both proteins are expressed in *S. aureus* and if both are required for promoting bacterial adhesion to fibronectin-coated surfaces, insertion mutations were isolated in each gene. A DNA fragment encoding tetracycline resistance was inserted into *fnbA* and a fragment encoding erythromycin resistance was inserted into *fnbB*. A double *fnbA fnbB* mutant was also constructed. The *fnbA* and *fnbB* single mutants showed no significant reduction in their adhesion to polymethylmethacrylate coverslips that had been coated *in vitro* with fibronectin. However, the double mutant was completely defective in adhesion. Monospecific antibodies directed against the non-conserved N-terminal regions of both proteins confirmed the lack of expression of FnBPs in the mutant strains. Wild-type *fnbA* and *fnbB* genes cloned seperately on a multicopy plasmid were each able to restore fully the adhesion-defective phenotype of the 8325-4 *fnbA fnbB* mutant. This demonstrates that both *fnb* genes are expressed in *S. aureus* and that both contribute to the ability of strain 8325-4 to adhere to fibronectin-coated surfaces. The double mutant was also defective in adhesion to coverslips that had been removed from tissue cages implanted subcutaneously in guinea-pigs, which suggests that fibronectin is important in promoting attachment of *S. aureus* to biomaterial *in vivo*.**

Received 21 February, 1995; revised 19 May, 1995; accepted 23 May, 1995. †Present address: Institute of Biosciences and Technology, Texas Medical Centre, 2121 W. Holcombe Blvd., Houston, Texas 77030-3303, USA. *For correspondence. E-mail tfoster@mail.tcd.ie; Tel. (1) 6082014; Fax (1) 6799294.

Introduction

Staphylococcus aureus is a major cause of infections associated with indwelling catheters and cardiovascular and orthopaedic devices (Maki, 1982; Kristinsson, 1989). Adhesion of bacteria to implants is a crucial step in initiating device-related infections (Vaudaux *et al.*, 1984a; 1989). Once bound, the bacteria form a biofilm and are difficult to eradicate by phagocytosis. In addition, they become impervious to the action of antibiotics. As well as damaging the tissue surrounding the implant, bacteria can disseminate and cause abscesses, endocarditis and septicaemia.

S. aureus is thought to attach to implants by adhesion to host plasma and matrix proteins such as fibrinogen and fibronectin that are rapidly deposited on the surface of the foreign body (Kochwa *et al.*, 1977; Cottonaro *et al.*, 1981). Fibrinogen is the major component of the deposited host proteins but is subject to plasmin degradation and as a result becomes unable to promote bacterial attachment (Vaudaux *et al.*, 1993). In contrast, fibronectin is a relatively minor component. However, despite being degraded by proteolysis it retains the ability to promote bacterial attachment (Vaudaux *et al.*, 1993). Adhesion to fibronectin may also play a role in *S. aureus* infections in the absence of implants, e.g. in surgical wound infection and invasive endocarditis in humans and also in ruminant mastitis (Hamill, 1987). Kuypers and Proctor (1989) reported the isolation of a low-level-fibronectin-binding mutant of *S. aureus* 879R4S, generated by Tn*918* mutagenesis. This mutant had a reduced ability to adhere to traumatized rat heart valves, indicating that fibronectin binding is important *in vivo*.

Two distinct but related fibronectin-binding protein genes have been cloned from *S. aureus* 8325-4 (Signas *et al.*, 1989; Jonsson *et al.*, 1991). The two genes *fnbA* and *fnbB* are separated by 682 bp and probably arose by gene duplication. The predicted proteins, FnBPA and FnBPB, are quite distinct in the N-terminal region A (45% identical) whereas region D1–D3 (fibronectin-binding domain) and regions W and M (wall-spanning and membrane anchor) are 95% identical. (The domain organization of the FnBPs is summarized in Fig. 1). When cloned in *Escherichia coli*, both genes expressed proteins with fibronectin-binding activity and it is assumed that both are

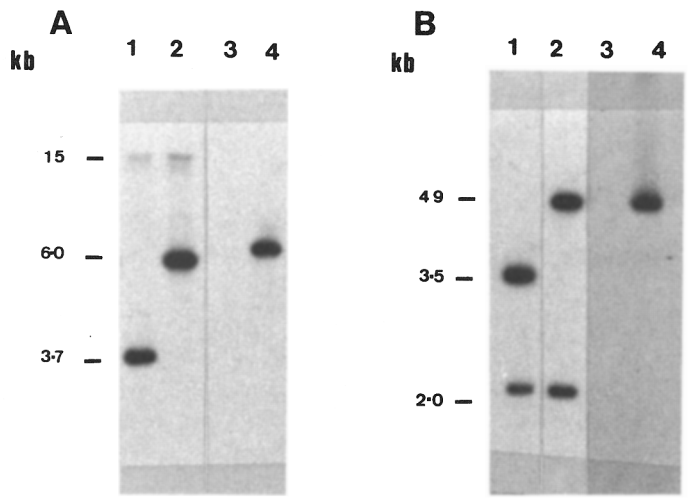
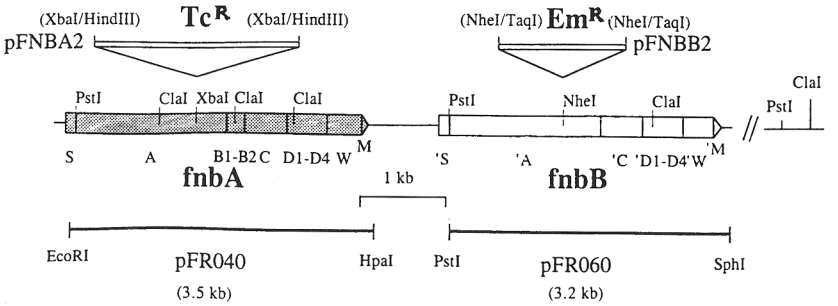


Fig. 1. Southern blot hybridization analysis of the *fnb* loci of *S. aureus* strains RN4220 and DU5922 (*fnbA*::Tc^R) and DU5923 (*fnbB*::Tc^R). Chromosomal DNA of *S. aureus* strains was digested with *Pst*I, (A) or *Cla*I (B) and electrophoresed on 0.8% agarose gel, transferred onto a nylon membrane and probed with α-^{32}P-labelled pFR040 and pCW59 (A) or pFR060 and pE194 (B).
A. *S. aureus* strains RN4220 (lane 1) and DU5922 (lane 2) were probed with pFR040, and RN4220 (lane 3) and DU5922 (lane 4) were probed with pCW59.
B. Strains RN4220 (lane 1) and DU5923 (lane 2) were probed with pFR060. Strains RN4220 (lane 3) and DU5923 (lane 4) were probed with pE194. The map shows the structure of the *fnb* locus of *S. aureus* strain 8325-4. S, A, B1–B2, C, D1–D4, W and M are domains in the FnBPA protein. Relevant restriction sites are indicated. The positions of the *fnbA*::Tc^R and *fnbB*::Em^R mutations are indicated.



expressed in *S. aureus* and contribute to the fibronectin-binding phenotype.

Fibronectin is a component of the extracellular matrix and of blood clots. It is a dimeric glycopeptide composed of a series of domains comprising different combinations of type I, type II and type III modules (Ruoslahti, 1988). The N-terminal 27 kDa peptide formed by plasmin degradation (Proctor, 1987) can bind to *S. aureus* cells with the same kinetics as native fibronectin (K_d 1.8 nM) (Bozzini *et al.*, 1992) and it can also promote bacterial attachment to coated surfaces. The five type I modules in the N-terminus of fibronectin are implicated in the binding reaction with *S. aureus* (Sottile *et al.*, 1991). Site-directed mutagenesis of a recombinant truncated fibronectin gene expressed by a mammalian cell line in culture showed that each of the five type I modules is important in the binding reaction. A second binding domain with a K_d of 10 nM has been located in the single type III heparin-binding module number 14 (Bozzini *et al.*, 1992). This domain may only be expressed in fibronectin that has taken on a fibrillar conformation when associated with surfaces and may act synergistically with N-terminal type I sites for the binding of *S. aureus* cells to fibrillar fibronectin.

The objectives of this research were to examine the expression of the *fnb* genes of *S. aureus* 8325-4, to establish if both FnBPA and FnBPB proteins are expressed by this organism, and to determine if both are required for adhesion to fibronectin-coated surfaces. The promoters of both *fnbA* and *fnbB* genes were located and their expression was examined using fusions to a luciferase reporter. Specific mutants with insertions in *fnbA* and *fnbB* were isolated and were tested for adhesion to polymethylmethacrylate (PMMA) coverslips coated *in vitro* with fibronectin. The ability of the cloned *fnbA* and *fnbB* genes to complement the *fnbA fnbB* double mutant was tested. Strains were also tested for adhesion to coverslips taken from tissue cages that had been implanted in guinea-pigs.

Results and Discussion

S. aureus *mutants defective in* fnbA

An insertion mutation in the *fnbA* gene was constructed by cloning a fragment from pCW59 which confers tetracycline resistance into the *Xba*I site in region A of *fnbA* in plasmid pFR040 located 5′ to the region that encodes

fibronectin-binding determinants and wall attachment. A *ts* shuttle plasmid was constructed (pFNBA2) by combining pFNBA1 with pTS2 (*ts*, chloramphenicol resistant (Cm^R)) which allowed allelic replacement recombinants in the *fnbA* gene of *S. aureus* RN4220 to be isolated. The chromosomal *fnbA*::Tc^R mutation was verified by Southern hybridization. Figure 1 shows chromosomal DNA of *S. aureus* strains RN4220 and DU5922 digested with *Pst*I and probed with radiolabelled pFR040 (Fig. 1A, lanes 1 and 2) and pCW59 (Fig. 1A, lanes 3 and 4). The pFR040 (*'fnbA*) probe hybridized strongly to a *Pst*I fragment of 3.7 kb in *S. aureus* RN4220 and to a fragment of 6.0 kb in *S. aureus* DU5922. The weakly hybridizing bands of >15 kb in Fig. 1A (lanes 1 and 2) react because of the homology between pFR040 and a large *Pst*I fragment carrying the *fnbB* gene and sequences 3′ to the *fnb* locus (Fig. 1). There is no signal in Fig. 1A (lane 3), showing that pCW59 does not hybridize to *S. aureus* RN4220 genomic DNA. The pCW59 probe hybridized to a fragment of 6.0 kb (Fig. 1A, lane 4) in *S. aureus* DU5922. This shows that the *fnbA* gene had been disrupted by insertion of the 2.35 kb tetracycline-resistance (Tc^R) determinant in *S. aureus* strain DU5922.

S. aureus *mutants defective in* fnbB

An insertion mutation in the *fnbB* gene was constructed by cloning a fragment from pE194 which confers erythromycin resistance (Em^R) into the *Nhe*I site in region A of *fnbB*. Plasmid pFNBA2 is a *ts* shuttle plasmid constructed in order to introduce the *fnbB*::Em^R mutation into the chromosome of *S. aureus*. *fnbB*::Em^R mutants of RN4220 were identified following elimination of the *ts* pFNBB2. The structure of the *fnb* locus was examined by Southern hybridization. Figure 1B shows chromosomal DNA of *S. aureus* strains RN4220 and DU5923 digested with *Cla*I and probed with pFR060 (Fig. 1B, lanes 1 and 2) and pE194 (Fig. 1B, lanes 3 and 4). Plasmid pFR060 (*'fnbB*) hybridized to *Cla*I fragments of 2.0 kb and 3.5 kb in *S. aureus* RN4220 (Fig. 1B, lane 1) while pFR060 hybridized to *Cla*I fragments of 2.0 kb and 4.9 kb in *S. aureus* DU5923. The 2.0 kb band carries the 3′ part of the *fnbB* gene and some 3′ non-coding sequence that is unaffected by the insertion. However, the 4.9 kb fragment is due to insertion of the 1.4 kb Em^R marker into the 3.5 kb *Cla*I fragment carrying 5′ sequences of *fnbB*. Cross-hybridization with *Cla*I fragments from *fnbA* were not detected because they were too small to be retained on the gel. There is no signal in Fig. 1B (lane 3), showing that pE194 does not hybridize to *S. aureus* RN4220. Plasmid pE194 hybridized to the Em^R marker on a fragment of 4.9 kb in *S. aureus* DU5923 (Fig. 1B, lane 4). This shows that *fnbB* had been disrupted by insertion of the Em^R marker in *S. aureus* strain DU5923.

Analysis of fnbA *and* fnbB *promoters*

Two putative promoters were suggested for each of the *fnbA* and *fnbB* genes of *S. aureus* 8325-4 (Signas *et al.*, 1989; Jonsson *et al.*, 1991). Primer extension analysis (data not shown) of RNA from 8325-4 carrying multicopy *fnb* plasmids pFNBPA4 or pFNBPB4 showed that both *fnbA* and *fnbB* transcripts terminated at a T residue situated 50 bp 5′ to the GTG start codon of the genes. The expression of the promoters was investigated by using bacterial luciferase (*lux*) as a reporter enzyme. Plasmids pFNBPA6 and pFNBPB6 allowed expression of the luciferase reporter driven by the *fnbA* and *fnbB* promoters, respectively (Fig. 2). The *fnbB* promoter expressed a threefold higher level of luciferase than the *fnbA* promoter. This might be explained by the −10 region of the *fnbB* promoter showing a closer resemblance to the consensus (5/6) than to the *fnbA* promoter (4/6).

Analysis of FnBP expression

In order to characterize the proteins expressed in *S. aureus*, specific antibodies were generated to the N-terminal non-conserved regions of FnBPA and FnBPB. These antibodies were used in Western immunoblots to examine expression of FnBPs by the wild-type 8325-4 and *fnb* mutant strains and strains carrying an *fnb*$^+$ gene on a multicopy plasmid.

Examination of supernatants of exponentially growing

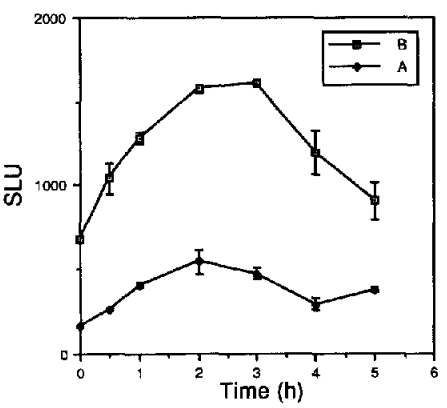


Fig. 2. Analysis of expression of the promoters of *fnbA* and *fnbB*. The promoters and 5′ flanking sequences of *fnbA* and *fnbB* were PCR-amplified and translationally coupled to *lux*, generating plasmids pFNBA6 and pFNBB6. At time = 0, saturated cultures of *S. aureus* 8325-4 and RN6911 harbouring the plasmids pFNBA6 and pFNBB6 were diluted 1:100 into fresh medium and growth was continued at 30°C with shaking. At regular intervals, 1 ml samples were removed and assayed for luminescence. Growth of the cultures was monitored by measuring the absorbance at 540 nm. The specific light units (SLU) for each time point were calculated by dividing the relative light units (as determined using a BioOrbit 1250 luminometer) by absorbance at 540 nm. Each data point represents the average of three experiments. The standard deviations are shown by error bars.

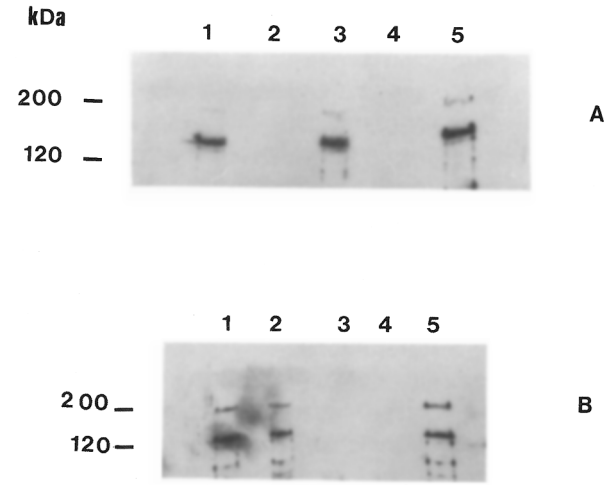


Fig. 3. Western blot of TCA-precipitated supernatant fractions of *S. aureus* 83254, DU5881, DU5882, DU5883, DU5883(pFNBA4) and DU5883(pFNBB4). Aliquots containing 100 μg of protein precipitated from late exponential cultures (OD_{540nm} = 1.0) were separated on 7.5% SDS–PAGE and transferred to a nitrocellulose filter.
A. Lane 1, 8325-4; lane 2, DU5881; lane 3, DU5882; lane 4, DU5883; lane 5, DU5883(pFNBA4) probed with anti-FnBPA and protein A–peroxidase.
B. Lane 1, 8325-4; lane 2, DU5881; lane 3, DU5882; lane 4, DU5883; lane 5, DU5883(pFNBB4) probed with anti-FnBPB and protein A-peroxidase.

cultures of *S. aureus* by Western blotting revealed proteins that cross-reacted with anti-FnBP serum (Fig. 3). Anti-FnBPA reacted strongly with a protein with an apparent molecular mass of 140 kDa and weakly with a protein of >200 kDa in samples from *fnbA*$^+$ strains. Similarly, anti-FnBPB reacted with proteins of >200 kDa (weakly) and 130 kDa (strongly) in samples from *fnbB*$^+$ strains. The same pattern of expression of FnBPs was seen in lysostaphin extracts of cells except that the higher molecular mass form (>200 kDa) of FnBPA and FnBPB was only detected in strains harbouring multicopy complementing plasmids (data not shown).

We suggest that the >200 kDa proteins are the native forms of FnBPA and FnBPB and that the 140 kDa and 130 kDa proteins are truncated derivatives. In *E. coli*, both *fnbA*$^+$ and *fnbB*$^+$ plasmids expressed immunoreactive proteins of >200 kDa along with many smaller bands (data not shown). Both FnBPA and FnBPB appear to be very susceptible to proteolytic degradation in *S. aureus*. The molecular masses of FnBPA and FnBPB predicted from their nucleotide sequences are 108 kDa and 98 kDa, respectively (Signas *et al.*, 1989; Jonsson *et al.*, 1991). The high proline content within the wall-spanning region Wr may account for their anomalous migration in SDS–PAGE. It is also possible that the >200 kDa proteins are dimers that are resistant to boiling in a buffer containing SDS and β-mercaptoethanol.

Bacterial adhesion of S. aureus *to* in vitro *fibronectin-coated coverslips*

In order to determine if both FnBPA and FnBPB are needed for bacterial attachment to fibronectin-coated surfaces, 8325-4 mutants defective in *fnbA* or *fnbB* alone, and the double *fnbA fnbB* mutant, were compared in an *in vitro* adherence assay. In addition, the wild-type *fnbA* and *fnbB* genes in multicopy shuttle plasmids, pFNBA4 and pFNBB4, were introduced into the *fnbA fnbB* double mutant for complementation tests.

The wild-type 8325-4 strain adhered to solid-phase fibronectin on gelatin–PMMA coverslips in a concentration-dependent fashion (Fig. 4A). Similar dose-responses, notwithstanding minor differences which did not reach statistical significance, were observed with the *fnbA* and *fnbB* single mutants (Fig. 4A). Coverslips which did not contain fibronectin (gelatin controls) bound $< 6 \times 10^3$ cfu. When average values of quantitative adhesion of wild-type and single *fnb* mutants were compared by statistical analysis, no significant differences were found despite the apparent lower adherence of the *fnbB* mutant. In

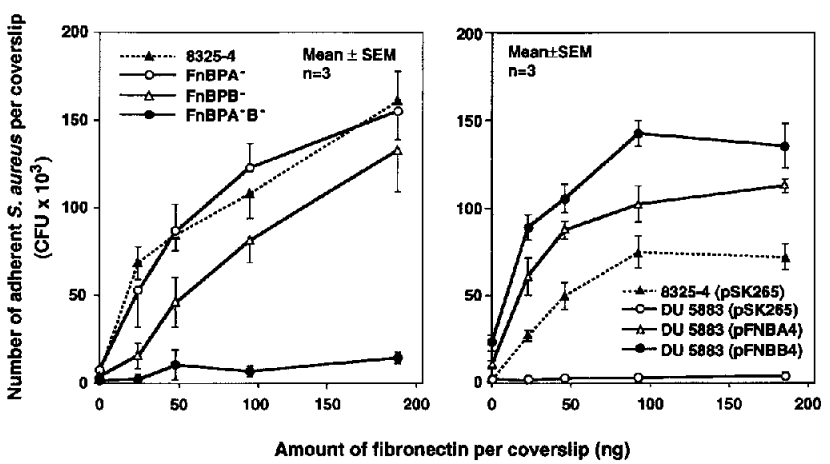


Fig. 4. Adhesion to fibronectin-coated coverslips.
A. *S. aureus* 8325-4, DU5881, DU5882 and DU5883.
B. *S. aureus* 8325-4(pSK265), DU5883(pSK265), DU5883(pFNBA4) and DU5883(PFNBB4). Fibronectin-coated (ranging from 0.125–1 μg ml^{-1}) gelatin–PMMA coverslips were incubated with 4×10^6 cfu of [^{3}H]-thymidine-labelled *S. aureus* per ml and, after rinsing, the number of attached bacteria was estimated from radioactive counts.

contrast to single mutants, the *fnbA fnbB* double mutant (DU5883) showed a complete loss of dose-dependent attachment to surface-bound fibronectin (Fig. 4A). This demonstrates that both FnBPA and FnBPB are expressed in *S. aureus* and that both contribute significantly to adherence to fibronectin-coated surfaces.

The ability of pFNBA4 and pFNBB4 to restore the adhesive phenotype of the *fnbA fnbB* double mutant by complementation was tested *in vitro* (Fig. 4B). *S. aureus* 8325-4 and DU5883 transformed with pSK265 displayed the same binding characteristics (Fig. 4B) as their plasmid-free counterparts shown in Fig. 4A. Not only did pFNBA4 and pFNBB4 restore the adhesion-defective phenotype of DU5883 but each individual plasmid also conferred a significantly ($P<0.05$) higher level of adhesion to fibronectin-coated coverslips than the parental 8325-4; this could be the result of a gene-dosage effect. The differences in the average dose-responses of adherence promoted by pFNBPB4 compared to pFNBPA4 did not reach statistical significance.

Bacterial adhesion to explanted coverslips

Two previous studies (Delmi *et al.*, 1994; Vaudaux *et al.*, 1984a) have demonstrated the role of fibronectin in promoting *in vivo* attachment of *S. aureus* or *Staphylococcus epidermidis* to coverslips explanted from guinea-pigs. Using this experimental system, we tested in parallel the adhesion to explanted coverslips of strain 8325-4 and its *fnbA fnbB* double mutant (DU5883). Figure 5 shows that compared to the parent strain, the double mutant is severely impaired in its ability to attach to *in vivo* coated coverslips ($P<0.002$). This demonstrates that fibronectin deposited on biomaterial from a foreign-body implant is an important determinant of bacterial attachment.

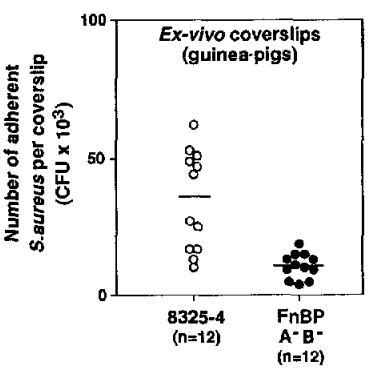


Fig. 5. Adhesion of *S. aureus* 8325-4 and DU5883 to *ex vivo* coverslips. Tissue cages containing PMMA coverslips were implanted subcutaneously into guinea-pigs for one month. Following removal and rinsing, 1 ml aliquots of ^{3}H-labelled *S. aureus* 8325-4 or DU5883 were incubated with the coverslips. Washed slides were evaluated for bacterial attachment by scintillation counting.

Conclusions

(i) Studies with site-specific *fnbA* and *fnbB* insertion mutants of *S. aureus* 8325-4 showed that both genes are expressed in *S. aureus* and that both FnBPA and FnBPB proteins contribute to the ability of the organism to adhere to fibronectin-coated surfaces *in vitro*.
(ii) The adherence-defective phenotype of the *fnbA fnbB* double mutant was fully restored by either wild-type *fnbA* or *fnbB* genes expressed from multicopy plasmids. A higher level of adherence was observed with strains bearing a multicopy complementing plasmid.
(iii) The *fnbA fnbB* double mutant was completely deficient in adherence to coverslips explanted from subcutaneous tissue cages in guinea-pigs, confirming that fibronectin deposited on a foreign-body implant is an important determinant of bacterial adhesion.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Bacterial growth media and antibiotics

E. coli strains harbouring plasmids were routinely grown in L-broth and L-agar (Miller, 1972) incorporating antibiotics as follows: ampicillin (Ap), 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; erythromycin, 150 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}, where appropriate. *S. aureus* strains were grown in trypticase soy broth (TSB) or agar incorporating antibiotics as follows: tetracycline, 3 μg ml^{-1}; erythromycin, 10 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}, where appropriate.

Manipulation of DNA

DNA-modifying enzymes were purchased from New England Biolabs or from Promega and were used according to the manufacturers' instructions. DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1987). 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 random-primer labelled with [α-^{32}P]-dATP (Amersham) using Promega's Prime-A-Gene system, as instructed by the suppliers.

Transfer of DNA

Plasmid DNA was transformed into *E. coli* TB1 cells made competent by $CaCl_2$ treatment and into *S. aureus* by electroporation. Transduction in *S. aureus* was performed by the method of Asheshov (1966) using bacteriophage 85.

Plasmid constructions

Plasmids for allelic replacement mutagenesis. Plasmids

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Relevant genotype/Host	Relevant phenotype/Markers	Properties	Source/Reference
Strain				
Escherichia-coli				
TB1	*ara thi rspL hspR* Δ*lac-pro*$_{XIII}$ (φ80*lacIZ*Δ*M15*)		Host for detecting chimaeric pUC plasmids	Yanisch-Perron *et al.* (1985)
Staphylococcus aureus				
RN4220	*fnbA*$^+$ *fnbB*$^+$	FnBPA$^+$ FnBPB$^+$	Mutant of 8325-4 capable of stably maintaining shuttle plasmids	Kreiswirth *et al.* (1983)
DU5922	*fnbA*::Tc^R	FnBPA$^-$	Mutant of RN4220 defective in expression of FnBPA	This work
DU5923	*fnbB*::Em^R	FnBPB$^-$	Mutant of RN4220 defective in expression of FnBPB	This work
8325-4	*fnbA*$^+$ *fnbB*$^+$	FnBPA$^+$ FnBPB$^+$	NCTC 8325 cured of prophages. Plasmid free	Novick (1963)
DU5881	*fnbA*::Tc^R	FnBPA$^-$	Mutant of 8325-4 defective in expression of FnBPA	This work
DU5882	*fnbB*::Em^R	FnBPB$^-$	Mutant of 8325-4 defective in expression of FnBPB	This work
DU5883	*fnbA*::Tc^R *fnbB*::Em^R	FnBPA$^-$ FnBPB$^-$	Mutant of 8325-4 defective in expression of FnBPA and FnBPB	This work
Plasmid				
pFR040	*E. coli*	Ap^R	3.5 kb insert of *fnbA* lacking 147 bp from 5′ sequence, in MCS of pUC18	Jonsson *et al.* (1991)
pFR060	*E. coli*	Ap^R	3.2 kb insert of *fnbB* lacking 113 bp from 5′ sequence, in MCS of pUC18	Jonsson *et al.* (1991)
pFR001	*E. coli*	Ap^R	*fnbA* and *fnbB* lacking 1.1 kb from 3′ sequence, in *Bam*HI site of pBR322	Signas *et al.* (1989)
pFNBA1	*E. coli*	Ap^R, Tc^R	2.35 kb *Hin*dIII fragment of pCW59 cloned into *Xba*I site in *fnbA* gene	This work
pFNBB1	*E. coli*	Ap^R, Em^R	1.4 kb *Taq*I fragment of pE194 cloned into *Nhe*I site in *fnbB* gene	This work
pCW59	*S. aureus*	Cm^R, Tc^R	Carries 2.35 kb Tc^R *Hin*dIII fragment	Wilson *et al.* (1981)
pE194	*S. aureus*	Em^R	1.4 kb Em^R *Taq*I fragment	Horinouchi and Weisblum (1982)
pTS2	*S. aureus*	Cm^R, *ts rep*	Derived from pTV1*ts*. MCS from pBluescript	C. O'Connell (personal communication); Youngman (1987)
pFNBA2	Shuttle	Ap^R, Tc^R, Cm^R, *ts rep*	pTS2 cloned into *Eco*RI site of pFNBA1	This work
pFNBB2	Shuttle	Ap^R, Em^R, Cm^R, *ts rep*	pTS2 cloned into *Eco*RI site of pFNBB1	This work
pGEX-KG	*E. coli*	Ap^R	GST fusion-protein expression vector	Guan and Dixon (1991)
pGEX-KG1	*E. coli*	Ap^R	GST–FnBPA fusion-protein expression vector	This work
pGEX-KG2	*E. coli*	Ap^R	GST–FnBPB fusion-protein expression vector	This work
pGEM-7Z(f)+	*E. coli*	Ap^R	Cloning vector	Promega
pFNBA3	*E. coli*	Ap^R	pGEM-7Zf(+) with *fnbA* gene, cloned from pFR001 on an *Sph*I–*Hpa*I fragment between *Sph*I and *Sma*I	This work
pSK265	*S. aureus*	Cm^R	*S. aureus* replicon	Jones and Khan (1986)
pFNBA4	Shuttle	Ap^R, Cm^R	pSK265 (cut with *Pst*I) cloned into pFNBA3 at *Nsi*I site	This work
pBluescriptKS	*E. coli*	Ap^R	Cloning vector	Short *et al.* (1988)
pFNBB3	*E. coli*	Ap^R	pBluescript KS with PCR-amplified *fnbB* gene at the *Eco*RI site	This work
pFNBB4	Shuttle	Ap^R, Cm^R	pSK265 cloned into pFNBB3 at *Xba*I site	This work
pSB327	Shuttle	Ap^R, Cm^R	Promoterless *lux* vector	Hill *et al.* (1993)
pFNBA6	Shuttle	Ap^R, Cm^R	PCR-amplified *fnbA* promoter region cloned *Eco*RI–*Sma*I-cleaved pSB327. *lux* fusion	This work
pFNBB6	Shuttle	Ap^R, Cm^R	*fnbB* promoter region cloned into *Eco*RI–*Sma*I-cleaved pSB327. *lux* fusion	This work

Ap, ampicillin; Tc, tetracycline, Em, erythromycin; Cm, chloramphenicol. MCS, multiple cloning site; *ts rep*, temperature-sensitive replication system; GST, glutathione S-transferase.

pFR040 and pFR060 are pUC18 derivatives which carry the *fnbA* and *fnbB* genes from *S. aureus* 8325-4, respectively, but lack the promoters and the 5′ coding sequences (′*fnbA* and ′*fnbB*). These were kindly supplied by Dr Martin Lindberg (Uppsala, Sweden). Mutations were constructed in the *fnbA* and *fnbB* genes by insertion of short DNA fragments encoding antibiotic resistance into a restriction site within the coding sequence carried by these plasmids. Temperature-sensitive shuttle plasmids were then constructed in order to allow the mutations to be introduced into the chromosomal *fnbA* and *fnbB* genes of *S. aureus* by allelic replacement. The plasmids were constructed as follows.

Plasmid pFR040 (′*fnbA*) was partially digested with *Xba*I and treated with DNA polymerase I Klenow fragment. This was ligated to the 2.35 kb *Hin*dIII fragment of pCW59 (encodes resistance to tetracycline), which had also been treated with the Klenow enzyme and transformed into *E. coli* TB1. An Ap^R Tc^R transformant with the correct structure (pFNBA1) was kept.

Plasmid pFR060 (′*fnbB*) was digested with *Nhe*I and treated with DNA polymerase I Klenow fragment. This was ligated to the 1.35 kb *Taq*I fragment of pE194 (encodes erythromycin resistance), which had also been treated with the Klenow enzyme and transformed into *E. coli* TB1. An Ap^R Em^R transformant with the correct structure (pFNBB1) was kept.

Temperature-sensitive (*ts*) shuttle plasmids derived from pFNBA1 and pFNBB1 were then constructed by cloning the *ts S. aureus* replicon pTS2 (encodes chloramphenicol resistance) into unique *Eco*RI sites in each plasmid, forming pFNBA2 and pFNBB2, respectively. DNA was transformed into *E. coli* TB1 and Ap^R Cm^R transformants with the predicted structures (pFNBA2 and pFNBB2) were kept (Fig. 6, A and B).

Plasmids for complementation tests. The wild-type *fnbA* and *fnbB* genes of *S. aureus* 8325-4 were cloned into multicopy shuttle plasmids as follows.

Plasmid pFR001 carries the entire 8325-4 *fnbA* gene and 1.1 kb of 5′ sequence. It was digested with *Sph*I and *Hpa*I to release this DNA plus 557 bases of 3′ flanking DNA. This was ligated to pGEM-7Zf(+) cut with *Sph*I and *Sma*I and transformed into *E. coli* TB1 to form pFNBA3. Plasmid pFNBA3 was digested with *Nsi*I and ligated to pSK265, a Cm^R *S. aureus* plasmid which had been digested with *Pst*I. An Ap^R Cm^R transformant of *E. coli* TB1 harbouring a plasmid with the predicted structure (pFNBA4, Fig. 6C) was kept.

The *fnbB* gene of *S. aureus* 8325-4 was amplified by the polymerase chain reaction (PCR) from chromosomal DNA using VENT DNA polymerase (New England Biolabs) and the oligonucleotides 5′-GCAGAATTCGTCGGCTTGAAATACGCTG-3′ (forward) corresponding to bases −427 to −408 5′ to the *fnbB* gene and 5′-GTAGAATTCACGCCTTCATAGTGTATTG-3′ (reverse), corresponding to bases 2917 to 2936 3′ to *fnbB*, with 30 temperature cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min 30 s. Each primer had a 9 bp 5′ extension including an *Eco*RI restriction site (underlined). The amplified PCR product which encoded the *fnbB* gene was cut with *Eco*RI and ligated to *Eco*RI-cleaved pBluescript. This was transformed into *E. coli* TB1 and transformants harbouring pFNBB3 were isolated. Plasmid pFNBB3 was cut with *Xba*I and ligated to *Xba*I-cleaved pSK265. Following

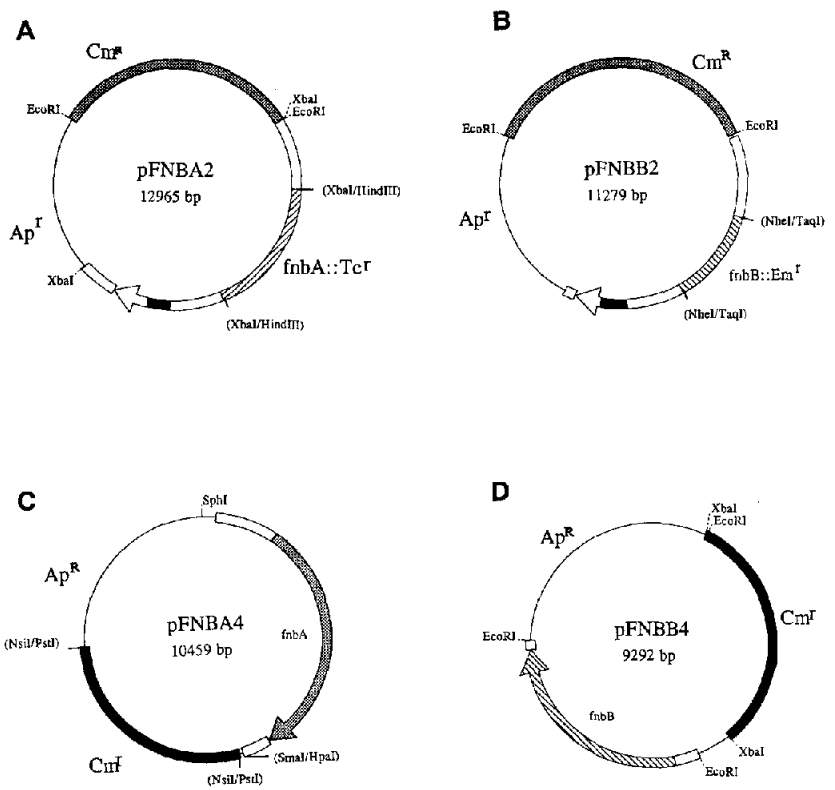


Fig. 6. Plasmids pFNBA2, pFNBB2, pFNBB4 and pFNBB4.
A. pFNBA2 carries the *fnbA* gene and some 3′ flanking DNA (open box and arrow) which has been disrupted by introduction of the tetracycline-resistance marker (slashed box) from pCW59. The solid box represents the D1–D4 repeat-encoding region. The shaded box is the 4.2 kb *ts S. aureus* plasmid pTS2. The thin line represents pUC18.
B. Plasmid pFNBB2. The open box represents the *fnbB* gene and some 3′ flanking sequence. The solid box represents the D1–D4 repeat-encoding region. The slashed box denotes the Em^R marker from pE194. The shaded box encodes the 4.2 kb *ts S. aureus* plasmid pTS2. The thin line represents pUC18.
C. Plasmid pFNBA4 is a shuttle plasmid derived from pGEM-7Zf(+) (Ap^R, thin line) and pSK265 (Cm^R, solid box). It carries the *fnbA* gene (shaded arrow) with some 5′ and 3′ flanking DNA (open boxes).
D. Plasmid pFNBB4 is a shuttle plasmid derived from pBluescript (Ap^R, thin line) and pSK265 (Cm^R, solid box). The shaded arrow denotes the *fnbB* gene with some 5′ and 3′ flanking sequences (open boxes). Relevant restriction sites are indicated.

transformation into *E. coli* TB1, an Ap^R Cm^R transformant with the predicted structure (FNBB4, Fig. 6D) was isolated.

Plasmids pFNBA4 and pFNBB4 were electrotransformed individually into *S. aureus* RN4220 selecting for Cm^R. They were then transduced into *S. aureus* DU5883 (*fnbA fnbB*).

Glutathione S-transferase fusions. In order to overexpress and purify proteins carrying parts of region A of FnBPA and FnBPB that were most divergent, DNA was amplified and linked to the glutathione S-transferase (GST) coding sequence of pGEX-KG. A fragment of the 8325-4 *fnbA* gene comprising bases 330–1674 was amplified by PCR using VENT DNA polymerase and primers 5′-GTGCCATGGCACAACCAG-AAATATAG-3′ (forward) and 5′- CGCAAGCTTCTGTGTGG-AATCAATGTC-3′ (reverse) with 30 temperature cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min 30 s. The primers has 9 bp extensions at their 5′ ends with restriction sites (underlined). The PCR product was digested with *Nco*I and *Hin*dIII, and ligated to pGEX-KG cut with the same enzymes to form pGEX-KG1(GST-FnBPA).

Bases 328 to 1489 of the *fnbB* gene of 8325-4 were PCR-amplified using primers 5′-GTGGAATTCTAGAAACTTCC-CGAGTTG-3′ (forward) and 5′-GCTCCATGGTCAAGTTC-ATAGGAGTAC-3′ (reverse) with restriction sites (underlined) incorporated in the 9 bp 5′ extensions. The PCR product was digested with *Eco*RI and *Nco*I and ligated to pGEX-KG cut with these enzymes forming pGEX-KG2 (GST-FnBPB). Both pGEX-KG1 and pGEX-KG2 expressed fusion proteins with the predicted molecular masses (76 and 70 kDa, respectively) after exponential cultures were induced with IPTG.

lux *fusions.* The promoter region of the *fnbA* gene was PCR-amplified from plasmid pFR001 using VENT DNA poymerase and the primers 5′-GTAGAATTCGTGCATG-AAGGAGATGG-3′ (F1), complementary to bases 551–569 of pBR322 flanking the *fnb*A insert and 5′-GATCCCGGGG-ATCTAAGATTGCTTTTC3′ (R1), complementary to bases 3–21 of *fnbA* (with two mismatches, one at position 4 (G → T) and the other at position 14 (T → C)) in a conserved part of region A of *fnbA* and *fnbB*. The *fnbB* promoter region was PCR-amplified from pFR001 with VENT DNA polymerase using primer 5′-GTAGAATTCGTCGGCTTGAAATACGGC-G-3′ (F2), complementary to bases −427 to −408 of *fnbB* in the intergenic region between *fnbA* and *fnbB*, and the same reverse primer (R1). The primers had restriction sites (underlined) incorporated in the 5′ 9 bp extensions. The PCR products generated with primers F1/R1 and F2/R1 were digested with *Eco*RI and *Sma*I and ligated to the *lux* reporter shuttle plasmid, pSB327, which had been cut with these enzymes. Transformants of *E. coli* TB1 (Ap^R Cm^R) harbouring the recombinant plasmids pFNBA6 and pFNBB6 were isolated. These plasmids were electrotransformed into *S. aureus* RN4220, selecting for Cm^R, and then transduced into strain DU5883.

Purification of FnBP fusion proteins and generation of antibodies

The GST-FnBPA and GST-FnBPB fusion proteins were expressed by adding IPTG (200 μM) to exponential cultures ($OD_{600\,nm}$ of 1.0) of *E. coli* (pGEX-KG1) and (pGEX-KG2) and incubating for 3 h. The proteins were purified by affinity chromotography using glutathione sepharose (Pharmacia). 40 μg of GST-FnBPA or GST-FnBPB was emulsified in an equal volume (500 μl) of complete Freund's adjuvant and injected subcutaneously into rabbits which had previously been screened for anti-FnBP antibodies. Following two boosts with 25 μg of protein in Freund's incomplete adjuvant at 14 day intervals, the rabbits were sacrificed by cardiac puncture and bled out. Antibodies were isolated from the serum (Owen, 1985) and IgG was purified by affinity chromatography with Protein A-sepharose (Pharmacia).

Construction of S. aureus fnbA::Tc^R *and* fnbB::Em^R *mutants by allelic replacement*

The *ts* shuttle plasmids pFNBA2 and pFNBB2 were electrotransformed into *S. aureus* RN4220, selecting for Tc^R or Em^R, respectively at 30°C. Temperature sensitivity of the plasmids was checked by measuring the efficiency of plating on Tc or Em agar at 30°C relative to 43°C (2×10^{-2} for FnBPA2 and 4×10^{-3} for pFNBPB2). Recombinants in which the *fnbA*::Tc^R or *fnbB*::Em^R mutations had replaced the wild-type chromosomal alleles were selected as follows. Cultures of RN4220(pFNBA2) and RN4220(pFNBB2) were inoculated into TSB with Tc or with Em as appropriate and grown to stationary phase at 30°C. The cultures were diluted 1:100 into fresh TSB lacking antibiotic and incubated at 43°C. After growth to stationary phase, cultures were diluted 1:100 in fresh broth and the incubation was repeated. When plasmid carriage by cells in the population was reduced by 10^{-4}–10^{-5} dilutions were plated on Tc or Em agar as appropriate. Single colonies were replica-plated onto Cm to identify putative recombinants that had lost the marker carried by the plasmid. Southern hybridization was performed with chloramphenicol-sensitive (Cm^S) derivatives to confirm that allelic replacement had occurred.

The *fnbA*::Tc^R and *fnbB*::Em^R mutations were transduced from *S. aureus* RN4220 (DU5922 and DU5923) into strain 8325-4. Double mutants carrying both *fnbA*::Tc^R and *fnbB*::Em^R were isolated by transducing the *fnbB*::Em^R mutation into 8325-4 *fnbA*::Tc^R (DU5881). Erythromycin-resistant transductants were isolated and scored for retention of the closely linked *fnbA*::Tc^R mutation. The *fnbA*::Tc^R mutation was lost in 20% of the Em^R transductants. The structures of the *fnb* loci of the transductants DU5881, DU5882 and DU5883 were verified by Southern hybridization (data not shown).

Luciferase assays

Luciferase activity was measured using a BioOrbit 1250 luminometer. Cultures were grown in 250 ml conical flasks containing 30 ml of TSB and incubated at 30°C with shaking. At regular intervals, 1 ml samples were removed, 10 μl of 1% (v/v) dodecyl aldehyde in ethanol was addded, and the samples were assayed for luminescence. The $OD_{540\,nm}$ of the cultures was determined at the time of sampling.

SDS–PAGE and Western blotting

SDS–PAGE was performed according to standard methods

(Laemmli, 1970) using 7.5% acrylamide gels. Aliquots (2.5 ml) of supernatants from late exponential growing cultures were precipitated for 30 min on ice with an equal volume of 25% trichloroacetic acid. Following washes with ice-cold acetone, the dried pellets were resuspended in Laemmli buffer, boiled, and aliquots containing 100 μg separated by SDS–PAGE. Proteins were transferred to nitrocellulose membranes (Millipore) using a semi-dry blotter (Bio-Rad Transblot SD). Filters were blocked in a 5% (w/v) solution of skim milk (Marvel) in PBS. Fibronectin-binding proteins were detected by incubation with anti-FnBPA (1:1000) or anti-FnBPB (1:1000) followed by protein A peroxidase (1:1000). The bound antibodies were detected using the ECL Western blotting reagent kit (Amersham).

Bacterial adhesion to in vitro *fibronectin-coated coverslips*

Purified human fibronectin (Chemicon) was dissolved in PBS at 1 mg ml^{-1} and stored at −70°C for a maximum of two months without any significant loss of activity. Solubilized fibronectin was checked for the absence of contaminants by SDS–PAGE and its concentration was measured spectrophotometrically (absorbance at 280 nm of 1.28; Vaudaux *et al.*, 1984b). An adherence assay (Vaudaux *et al.*, 1984b) modified to measure the ability of *S. aureus* to adhere to low levels of surface adsorbed fibronectin (Vaudaux *et al.*, 1993) was used. To optimize adsorption of fibronectin from concentrations below 1 μg ml^{-1}, the two-sided coverslips (8 × 9 mm) made of PMMA were pre-coated with gelatin (1 mg ml^{-1}). After rinsing in PBS, coverslips were incubated for 60 min at 37°C with low concentrations (from 0.125–1 μg ml^{-1}) of fibronectin, followed by rinsing in PBS (Vaudaux *et al.*, 1993). Surfaces were shown to be coated in a dose-dependent manner with amounts of fibronectin ranging from 22–190 ng per coverslip (15–132 ng cm^{-2}) when [^{3}H]-fibronectin (Spycher and Nydegger, 1986) was used. All strains tested for adherence were grown for 3 h at 37°C in Mueller–Hinton broth supplemented with 24 μCi ml^{-1} [^{3}H]-thymidine and with Cm when plasmids pSK265, pFNBA4 and pFNBB4 plasmids were present. For the adherence assay, gelatin–fibronectin coverslips were incubated with 4×10^6 cfu ml^{-1} [^{3}H]-thymidine-labelled *S. aureus* for 60 min at 37°C, as previously described (Vaudaux *et al.*, 1984b; 1993). The adherence medium was PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+} with 5 mg of human serum albumin per ml to prevent non-specific adherence (Vaudaux *et al.*, 1984b; 1993). Coverslips were rinsed, and the number of attached bacteria was estimated from radioactive counts (Vaudaux *et al.*, 1984b). Gelatin–PMMA coverslips not exposed to fibronectin were used as controls (Vaudaux *et al.*, 1984b; 1993).

Each dose-response curve is composed of means ± SEM of three experiments. The parental strain 8325-4 and its three *fnb* mutants were tested in parallel. Quantitative adhesion data were compared by one-way analysis of variance (ANOVA) and *t*-tests for comparisons of pairs of groups. Two-tailed significance levels with $P < 0.05$ with the Bonferroni Correction were considered as statistically significant.

Bacterial adhesion to explanted coverslips

Four tissue cages (which are perforated cylinders made from PMMA) were implanted subcutaneously into guinea-pigs as previously described (Vaudaux et al., 1984a; Zimmerli *et al.*, 1982). Each tissue cage contained three PMMA coverslips (Vaudaux *et al.*, 1984a). At one month after surgery, tissue cages and coverslips were explanted from the animals (Vaudaux *et al.*, 1984a; Zimmerli *et al.*, 1982). Following rinsing in PBS, explanted coverslips were incubated with ^{3}H-labelled cultures of either the parent strain of *S. aureus* 8325-4 or the *fnbA fnbB* double mutant and tested for adhesion using the same procedure as for the *in vitro* fibronectin-coated coverslips. Differences in quantitative adhesion were analysed by non-parametric testing (Mann–Whitney *U* test), with a *P* level of 0.05. Animal experiments in Dublin were sanctioned by the Minister of Health according to the Statutory Instrument Number 17 of 1994 entitled European Communities (Amendment of Cruelty of Animals Act, 1986). Official authorization in Geneva came from the Office Vetinaire Cantonal and was overseen by the Ethical Committee of the Faculty of Medicine.

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