Fibronectin-binding protein B variation in *Staphylococcus aureus*

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Abstract

**Background:** Fibronectin binding proteins A and B (FnBPA and FnBPB) mediate adhesion of *S. aureus* to fibrinogen, elastin and fibronectin. We previously identified seven different isotypes of FnBPA based on divergence in the fibrinogen- and elastin-binding A domains. The variation created differences in antigenicity while ligand binding functions were retained. Here, FnBPB variation was examined in both human and bovine isolates and compared to that of FnBPA.

**Results:** Seven different *fnbB* allelic variants were identified. Some strains that cluster by phylogenetic analysis contain different *fnbB* variants, whereas more divergent strains contain the same *fnbB* variant. The phylogeny of *fnbB* alleles does not match the phylogeny of *fnbA* alleles. Some FnBPA and FnBPB isotypes that are specified by human *S. aureus* strains are also found in bovine strains. The seven *fnbB* allelic variants encode seven distinct isotypes of the FnBPB A domain that are 61 to 85% identical in amino acid sequence. Variant amino acid residues were mapped on a three-dimensional model of the FnBPB A domain and were predicted to be surface-exposed. They are responsible for the antigenic diversity detected with polyclonal antibody and a monoclonal antibody raised against isotype I. Ligand binding by recombinant FnBPB N23 isotypes was compared by ELISA-based solid phase assays and surface plasmon resonance. Each bound to immobilized fibrinogen, elastin and fibronectin dose-dependently and saturably with similar affinities. Binding to fibronectin was surprising because the A domains do not contain any known motifs that mediate binding to fibronectin. This raises the possibility that the A domain of FnBPB contains a novel fibronectin binding motif that binds fibronectin by a novel mechanism.

**Conclusions:** Seven different isoforms of FnBPB A domain retain ligand-binding functions but are antigenically distinct. The variation in FnBPA and FnBPB occurs in human and bovine *S. aureus* strains and may act as an immune evasion mechanism. All seven isotypes of FnBPB are capable of binding fibronectin though none contain any known fibronectin-binding motifs. These results have implications for the development of vaccines or immunotherapeutics that target FnBPB.

Background

*Staphylococcus aureus* is a commensal that colonizes the moist squamous epithelium of the human anterior nares. Twenty percent of the population are permanently colonised while the remainder are colonized intermittently [1]. It is an important opportunistic pathogen that can cause superficial skin infections as well as invasive life-threatening conditions such as septic arthritis and endocarditis [2]. The success of *S. aureus* as a pathogen can in part be attributed to the expression of cell surface protein receptors designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that interact specifically with proteins present in the host plasma and extracellular matrix [3]. MSCRAMMs act as virulence factors that allow *S. aureus* to adhere to the surface of host cells and to damaged tissue and help it to avoid phagocytosis by neutrophils [4-6].

The fibronectin binding proteins (FnBPs) A and B of *S. aureus* are multifunctional MSCRAMMs which recognise fibronectin, fibrinogen and elastin [7-10]. FnBPA and FnBPB have considerable organization and sequence similarity and are composed of a number of distinct domains [7,9]. Figure 1 illustrates the domain organization of FnBPA and FnBPB of *S. aureus* strain 8325-4. Both pro-

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Most research on fibronectin-binding proteins has been performed with FnBPA from strain 8325-4. It was reported previously that the A domain of FnBPA of *S. aureus* strain 8325-4 comprising residues 37-544 bound to immobilized elastin and to fibrinogen (Figure 1) [8,10]. The A domain co-ordinates were revised recently and it was noted that the C-terminus of rFnBPA37-544 likely contained a single fibronectin-binding motif [16]. The ability of this protein to bind fibronectin was later confirmed [11]. In the same study, the revised A domain of FnBPA spanning residues 194-511 (Figure 1) was shown bind fibrinogen and elastin but not fibronectin. The minimum region of the FnBPA A domain needed for binding to fibrinogen and elastin is subdomains N23 (residues 194-511). The N1 sub-domain is not required for ligand binding [11].

The binding of FnBPs to fibronectin promotes the internalization of *S. aureus* into epithelial and endothelial cells which are not normally phagocytic [17,18]. FnBP-mediated invasion occurs through the formation of a fibronectin bridge between *S. aureus* and the α5β1 integrin [18]. This may promote bacterial dissemination from the bloodstream to internal organs and evasion of immune responses and antibiotics. This was convincingly demonstrated in a study of the role of FnBPA in experimental endocarditis where binding to both fibrinogen and fibronectin required. Binding of fibronectin was required for initial colonization of thrombi on damaged valves and while binding to fibronectin was required for the infection to spread [19].

FnBPA and FnBPB are encoded by two closely linked but separately transcribed genes, *fnbA* and *fnbB* [7,9]. While most strains contain both genes, some strains contain only *fnbA* [20]. In strain 8325-4, studies with site-specific *fnbA* and *fnbB* insertion mutants showed that either FnBPA or FnBPB mediated adherence to immobilized fibronectin but there was no significant difference in adherence between wild type strains and single *fnb* mutants [21]. However, studies with clinical isolates suggested that strain associated with invasive diseases are significantly more likely to have two *fnb* genes [20].

Seven variants (isotypes I-VII) of FnBPA were identified based on divergence in the amino acid sequences of the minimal ligand-binding N23 sub-domains [22]. Each FnBPA isotype retained ligand-binding activity but were antigenically distinct. Modelling the 3D structures showed that the amino acid variation occurred in surface-exposed residues and not in those involved in ligand-binding [22].

The initial aim of this study was to characterize the A domain of FnBPB and to determine the extent of variation in the A domain. It was discovered that the A domain of all FnBPB isotypes had the ability to bind to fibronectin by a novel mechanism.
**Results**

fnbB gene variation in *S. aureus* whole-genome sequences

Previously we reported that the A domain of FnBPA from strain P1 varied substantially from that of strain 8325-4, sharing only 73.5% amino acid identity [11]. We then identified seven variants of FnBPA A domain (isotypes I-VII) based on divergence in the minimal ligand-binding N23 sub-domain. Each recombinant N23 variant was shown to retain ligand-binding function but was antigenically distinct [22]. This prompted us to investigate variation in the A domain of the second fibronectin-binding protein, FnBPB.

DNA encoding the entire FnBPB A domain of strain P1 was amplified by PCR and sequenced. The deduced amino acid sequence was compared with that of strain 8325-4 and the overall identity was 80%. The A domain sequences of FnBPB from published *S. aureus* genomes were compared to determine if diversity in this domain is common amongst *S. aureus* isolates. All of the sequenced strains, except strain MRSA252 and the bovine strain RF122, contain genes encoding both FnBPA and FnBPB. Strains MRSA252 and RF122 both encode the FnBPB protein. The amino acid sequence of the A domain of FnBPB from *S. aureus* strains 8325-4, COL, USA300, Mu50, MSSA476, N315, MW2 and P1 were compared by pair-wise alignments and the identities calculated. Strains that are closely related and belonging to the same clonal complex are found to share identical A domains. However, comparison of A domain sequences of strains from different sequence types revealed that significant diversity exists. While subdomain N1 is highly conserved in all strains (94-100% amino acid identity) the N2 and N3 domains from unrelated isolates are significantly more divergent. Based on the sequences of the N23 subdomains, four variants of FnBPB (isotypes I-IV) were identified that share 61.1 - 80.6% amino acid identity (Table 1).

DNA hybridization analysis using fnbB isotype-specific probes

To determine the distribution of FnBPB A domain isotypes I - IV in *S. aureus* strains of different MLSTs and to identify any novel A domain isotypes, DNA hybridization was used with isotype-specific probes homologous to DNA specifying a portion of the highly divergent N3 sub-domain. DNA encoding the entire A domain was amplified with A domain flanking primers. PCR products were then spotted onto membranes and hybridized with the DIG-labelled type-specific probes. An example of the hybridization experiments with probes I - IV is shown in Figure 2. The probes were shown to be type-specific because each only hybridized to the appropriate control *fnbB* fragment (Figure 2A-D, top rows). *fnbB* DNA from *S. aureus* strains 2 (ST7), 114 (ST39), 233 (ST45), 304 (ST39), 3077 (ST17) and 3110 (ST12) did not hybridise to any of the probes, indicating that they may specify novel FnBPB isotypes or lack the *fnbB* gene.

**Identification of novel FnBPB isotypes (Types V, VI and VII)**

The *fnbB* gene fragments amplified from *S. aureus* strains 2 (ST7) 114 (ST39), 233 (ST45), 304 (ST39), 138 (ST30), 563 (ST37), 3077 (ST17) and 3110 (ST12) did not hybridise to probes specific for FnBPB isotypes I-IV. The *fnbB* gene fragments from these strains were cloned and sequenced, and the deduced A domain amino acid sequences were compared to the sequences of A domains of types I - IV. *S. aureus* strains 2 (ST7) and 3110 (ST12) specify a novel FnBPB A domain called isotype V (N23, 68.8 - 73.3% identical to isotypes I - IV). The A domains of strains 3077 (ST17) and 233 (ST45) are also different and are called isotype VI (N23, 66.0- 76.6% identical to types I - V) and isotype VII (N23, 66.2% - 85% identical to types I-VI) (Table 1). Strains 114, 563, 138 and 304 specify an identical A domain which is 92% identical to isotype II and is called isotype II* (Table 1).

**Phylogenetic analysis of FnBPB A domain isotypes I-VII**

Figure 3 shows a neighbour-joining phylogenetic tree which was constructed based upon the concatenated sequences of the seven housekeeping genes used for MLST analysis. As MLST reflects the evolution of the stable core genome [23], this tree describes the phyloge-
genes being closely linked. Alleles does not match that of respectively. This indicates that the phylogeny of isotype II [22]. In this study, these strains were found to carry fnbB alleles despite the two genes being closely linked.

Figure 2 FnBP A domain typing of S.aureus strains by dot blot hybridisation. DNA fragments coding for the entire A domain of fnbB were amplified by PCR from clinical S.aureus isolates. PCR products were spotted onto nitrocellulose membranes and probed with DIG-labelled probes specific for fnbB isotype I (A), II (B), III (C) and IV (D). fnbB DNA from strains 8325-4, N315, MSSA476 and P1 was used as control.

There are two major clusters of S. aureus strains studied here. It is separated into major clusters as was also shown previously in a detailed phylogenetic analysis of thirty diverse S.aureus isolates [24]. The FnBP A domain isotypes specified by each genotype (as predicted by DNA hybridisation or sequencing) are indicated. The phylogeny of fnbB alleles illustrated here does not correspond to that of the core genome as determined by MLST. For example, two strains that cluster together in Group 1 (ST49 and ST52) carry fnbB genes encoding isotype II, as do distant strains from Group 2 (ST5 and ST18). Conversely, clustered strains such as ST8 and ST97 from Group 2 contain fnbB genes encoding isotypes I and IV, respectively. Isolates belonging to the same ST (ST45) were found to specify different FnBPB isotypes (II and VII). These results suggest that fnbB alleles have dispersed by horizontal transfer, most likely by homologous recombination.

It has been recently reported that strains 116 (ST9) and 3077 (ST17) specify an identical FnBPA A domain called isotype II [22]. In this study, these strains were found to specify different FnBPA A domains, isotypes II and VI respectively. This indicates that the phylogeny of fnbB alleles does not match that of fnbA alleles despite the two genes being closely linked.

FnBP isotypes encoded by bovine S. aureus strains
We expanded the investigation into FnBP variation to include FnBPs from a variety of bovine S. aureus strains. Nineteen bovine isolates representing genetically unrelated strains were screened to determine if they specified the same FnBP isotypes as human strains. This strain collection included strain RF122, the genome of which has been sequenced [25]. RF122 contains only one fnb gene encoding FnBPA.

DNA encoding fnbA was amplified from the genomic DNA of each strain using generic A domain primers. PCR products hybridised to FnBPA probes specific for isotypes I, II, III or IV. Similarly fnbB DNA was amplified by PCR from the genomic DNA of all strains except RF122. These PCR products hybridised to FnBPB probes specific for isotype I, II, III, IV or V. These results indicate that the FnBP isotypes which are expressed by human strains are also specified by bovine strains. Furthermore, the results of this study suggest that the lack of fnbB in the genome of strain RF122 is not characteristic of all bovine strains. None of the strains tested specify FnBPA or FnBPB isotypes V, VI or VII.

Figure 4 shows a neighbour-joining phylogenetic tree which was constructed based on MLST data as described above. The FnBPA and FnBPB A domain isotypes specified by each genotype are included. The distribution of fnbB and fnbA variants does not correlate with the genetic relatedness of the strains as determined by MLST. The phylogeny of fnb alleles carried by bovine S. aureus isolates is therefore very similar to that of human strains.

Generation of 3D-models for FnBPB (N23) types I-VII and mapping the location of variant amino acid residues
Theoretical models of the structure of region A (N23) of FnBPB isotypes I-VII were generated based on the crystal structure of the equivalent domains of the S. aureus clumping factor ClfA. A ligand-binding trench is predicted to form between the N2 and N3 domains of FnBPB. C-terminal residues in sub-domain N3 are predicted to form the putative latching peptide. In each of the seven molecular models, the variant residues mapped to the surface of the protein while the residues within the predicted ligand-binding trench are highly conserved (Figure 5.). The predicted 3D structure obtained for FnBPB type I of strain 8325-4 and the predicted location of variant residues is shown in Figure 4. Residues 467-480 of FnBPB isotype I comprise the predicted latching peptide and are shown here in blue. In the crystal structure of the apo form of ClfA the latching peptide is folded over the N3 subdomain.

Antigenic variation: binding of antibodies to isotypes I-VII
We previously demonstrated that variation in the A domain of FnBPA resulted in proteins that are antigenically distinct. Here the ability of polyclonal anti-isotype I antibodies and a monoclonal anti-isotype I antibody to bind different recombinant FnBPA N23 isotypes was measured by ELISA. Polyclonal rabbit anti-isotype I antibodies had a 4-9 fold lower affinity at half maximum binding for isotypes II - VII compared to isotype I (Figure 6). This suggests that amino acid variation creates differ-
ences in surface-exposed epitopes on the A domain mole-
cule that affect immuno-crossreactivity. Mouse
monoclonal antibody 2E11 bound efficiently to isotype I
but showed little binding to isotypes II - VII as shown in
Figure 5. This suggests that the 2E11 epitope is only pres-
ent on isotype I.

Binding of FnBPB A domains isotypes I - VII to immobilized
ligands (ELISA)
Each recombinant N23 isotype bound to immobilized
fibrinogen and elastin in a dose-dependent and saturable
manner as shown in Figure 7. The estimated half maxi-
umum binding concentrations were 0.5 μM and 0.9 μM
respectively. These results confirm that the revised co-
oxidates of the N23 subdomain of region A of FnBPB
(isotypes I-VII) is sufficient for ligand-binding and that
subdomain N1 is not required.

Somewhat surprisingly, the seven N23 isotypes also
bound fibronectin dose-dependently and saturably with a
half-maximum binding concentration of 1.5 μM (Figure
7c). Recombinant FnBPA isotype I, which was previously
shown not to bind fibronectin, was a used was as a nega-
tive control. The ability of the FnBPB A domain proteins
to bind fibronectin was surprising because the amino
acid sequences do not contain any known fibronectin-
binding motifs.

Measuring the affinity of FnBPB A domain isotype I for
fibrinogen, elastin and fibronectin by surface plasmon
resonance
The results of the solid-phase binding assays suggested
that the A domain of FnBPB binds fibrinogen, elastin and
fibronectin with similar affinity. Estimated half maximal
binding concentrations were in the low micromolar
range. To verify these results, the affinities of rN23 iso-
type I for fibrinogen, elastin and fibronectin were mea-
sured using Surface Plasmon Resonance. Human
fibrinogen, elastin and fibronectin were immobilized
onto the surface of dextran chips. rN23 type I protein was
passed over the surface in concentrations ranging from

![Figure 3 Neighbour-joining tree based upon concatenated sequences of MLST alleles from human S. aureus strains](image)

MLST allele sequences representing each clinical strain studied here were used to generate a neighbour joining tree using MEGA 4. The A domain isotypes carried by strains of each MLST genotype, determined by sequencing and hybridization analysis, are indicated. The dashed line indicates the separation of the MLST genotypes into Groups 1 and 2, which is based on sequence data from MLST alleles and other unlinked loci (24). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (36,37). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (37). The evolutionary distances were computed using the Maximum Composite Likeli-
hood method (34) and are in the units of the number of base substitutions per site.
0.15 μM to 10 μM. The representative sensorgrams shown in Figure 8 have been corrected for the response obtained when recombinant protein was flowed over uncoated chips. The $K_D$ for the interaction with fibrinogen, elastin and fibronectin was 2 μM, 3.2 μM and 2.5 μM, respectively.

**Discussion**

The colonization of host tissue by *S. aureus* is an important factor in disease pathogenesis. *S. aureus* expresses on its cell surface a number of MSCRAMMs that promote colonization of diverse sites and contribute to virulence. Most *S. aureus* strains can express two distinct fibronec- tin-binding proteins (FnBPA and FnBPB). These two multifunctional MSCRAMMs both mediate adhesion to fibrinogen, elastin and fibronectin.

FnBPA and FnBPB are encoded by the two closely linked genes, *fnbA* and *fnbB* [20]. It has been reported that the *fnbA* and *fnbB* genes from 50 different strains representing the major MRSA clones found in Europe have undergone greater sequence divergence than genes encoding other surface proteins such as *clfA* and *clfB* [26]. Analysis of the *fnb* genes from published genome sequences showed that divergence was confined to the region encoding the N-terminal fibrinogen and elastin-binding A domains while the C-terminal fibronectin-binding motifs were highly conserved ([22] and this study). Our previous study identified seven isotypes of FnBPA based on divergence in the minimal ligand-bind- ing N23 sub-domain [22]. Each recombinant isotype was found to retain ligand-binding function but was antigenically distinct.

![Figure 4 Neighbour-joining tree based upon concatenated sequences of MLST alleles from bovine *S. aureus* strains.](image-url) MLST allele sequences representing each bovine-specific strain studied here were used to generate a neighbour joining tree using MEGA 4. The A domain isotypes carried by strains of each MLST genotype, as determined by hybridization analysis, are indicated. A gene encoding FnBPB is absent from the genome of strain RF122 (ST155). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [36]. The evolutionary distances were computed using the Maximum Composite Likelihood method [34] and are in the units of the number of base substitutions per site.
This study aimed to investigate the divergence in the A domain of FnBPB and to determine if variation in this region of the protein is widespread amongst S. aureus strains. The \textit{fnbB} gene sequences from sequenced S. aureus strains and strain P1 were compared. Four FnBPB variants (isotypes I-IV) were identified based on divergence in N23 sub-domains, which are 66-76% identical to one another.

In order to determine the distribution of FnBPB isotypes I-IV and to identify novel isotypes, type specific probes were generated and used to screen \textit{fnbB} DNA from a variety of clonal types using a well-characterized strain collection of human origin and human isolates where genomes have been fully sequenced [27]. Three novel FnBPB isotypes were identified (types V, VI and VII) which are 61.1\% - 85\% identical to isotypes I-IV. Phylogenetic analysis of FnBPB isotypes indicated that the phylogeny of \textit{fnbB} alleles does not correlate with the core genome as reflected by MLST. The evolution of \textit{S. aureus} has been predominantly clonal where alleles are 5- to 10-fold more likely to diversify by point mutations than by recombination [27]. The distribution of \textit{fnbB} alleles amongst different S. aureus lineages suggests, however, that recombination has been involved. Horizontal transfer by homologous recombination is likely to be responsible for the dispersal of genes encoding the same isotypes across strains of different phylogenies. The distribution of \textit{fnbA} alleles described in the study by Loughman et al does not match the distribution of \textit{fnbB} alleles described here [22]. Different combinations of FnBPA and FnBPB isotypes are specified by strains that cluster phylogenetically. For example, strains belonging to ST12 were shown to specify FnBPB Type V and FnBPA Type V. By contrast, strains belonging to ST13 specified FnBPB Type IV and FnBPA Type V. This suggests that the phylogeny of \textit{fnbB} alleles has evolved independently from that of \textit{fnbA} alleles and has involved separate recombination events despite the genes being closely linked.

Our study of FnBP variation in \textit{S. aureus} was extended here to include bovine \textit{S. aureus} strains. The genome of the bovine strain RF122 contains only the \textit{fnbA} gene but lacks \textit{fnbB}. Using generic primers, DNA encoding FnBPA and FnBPB was amplified from genomic DNA of nineteen bovine \textit{S. aureus} strains. The amplification of \textit{fnbB} DNA from these strains indicates that the lack of the \textit{fnbB} gene in strain RF122 is not common to all bovine \textit{S. aureus} strains. The \textit{fnbA} and \textit{fnbB} PCR products were subsequently probed with DNA probes specific for A domain isotypes specified by human \textit{S. aureus} strains. It was shown that bovine isolates specify the same isotypes of FnBPA and FnBPB as those specified by human isolates. The distribution of isotypes across the population of bovine strains tested was found to be uneven. No strains tested specified \textit{FnBPA} isotypes V, VI or VII or \textit{FnBPB} isotypes VI or VII. The majority of the strains tested were found to specify \textit{FnBPA} Type IV and \textit{FnBPB} Type II. Interestingly in the study of Loughman et al, \textit{FnBPA} Type II was found to be predominant in human clinical isolates [22]. It could be postulated that this difference in FnBPA isotype frequency reflects the differences in selective pressures posed by these two distinct host immune systems.

Further evidence for the role of recombination in the evolution of \textit{S. aureus} comes from the genome structure of ST239 strains which are composed of 557 kb of ST8...
DNA spliced into 2,220 kb of an ST30 strain [28]. Also, the gene for coagulase has undergone similar diversification as the fnb genes [29]. Recombination within coa genes encoding ten major isotypes has created novel subtypes and there is evidence for the same coa isotype being expressed by strains with different genetic backgrounds suggesting horizontal dissemination by homologous recombination [29].

A 3D molecular model of the N2 and N3 domains of FnBPB was generated based on the known structure of ClfA. Like the A domain of ClfA (and FnBPA) it is predicted that the N23 subdomain of FnBPB represents the minimal ligand binding region and a ligand binding trench is predicted to form between the N2 and N3 subdomains. Based on this model, it was shown that the majority of variant residues are located on the surface of the protein while residues that are predicted to be involved in ligand-binding are highly conserved. Amino acid sequence variation affected antibody recognition. Polyclonal antibodies against isotype I had reduced affinity for isotypes II – VII while a monoclonal antibody raised against isotype I had little or no affinity for all other isotypes. As with FnBPA isotypes, FnBPB sequence variation has created different epitopes on the A domains that affect immunocross-reactivity. This result is consistent with the predicted location of variant residues on the

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**Figure 7** Dose-dependent binding of rN23 isotypes I-IV to immobilised human fibrinogen (a), elastin (b) and fibronectin (c). Bound protein was detected with mouse anti-hexahistidine monoclonal antibody 7E8. rFnBPA N23 was used as a control in fibronectin-binding assays. Each assay was performed three times with similar results.
surface of the protein and not in regions involved in ligand binding. While most strains contain both genes, some strains contain only \textit{fnbA} [20]. Studies with site-specific \textit{fnbA} and \textit{fnbB} insertion mutants of strain 8325-4 have shown that either FnBPA or FnBPB can mediate adherence to immobilized fibronectin, but there was no difference in adherence between wild type strains and single \textit{fnb} mutants, indicating functional redundancy [21]. However, isolates associated with invasive diseases are significantly more likely to have two \textit{fnb} genes [20]. Combined antigenic variation in both FnBPA and FnBPB may be employed by \textit{S. aureus} to thwart the host immune responses during colonization or invasive infection. Interestingly, the diversity which occurs in the N2 and N3 subdomains of FnBPA and FnBPB does not occur in the N1 subdomain of either protein. For both FnBP proteins, the N1 subdomain is not required for ligand binding, similar to ClfA [13]. The A domain of both ClfA and another \textit{S. aureus} fibrinogen binding protein, clumping factor B (ClfB), are susceptible to cleavage by aureolysin at a SLAVA/SLAAVA motif located between subdomains N1 and N2 [30]. A SLAVA-like motif occurs in both FnBP proteins with S\textsubscript{177}ADVA\textsubscript{181} and S\textsubscript{144}TDVTA\textsubscript{149} present in FnBPA isotype I and FnBPB isotype I, respectively, which may render the A domains similarly susceptible to prote-

![Figure 8](http://www.biomedcentral.com/1471-2180/10/160/fig8)

**Figure 8** Dose-dependent binding of rFnBPB to fibrinogen (a), elastin (b) and fibronectin (c) as determined by Surface Plasmon Resonance. Human fibrinogen, elastin and fibronectin were immobilised onto the surface of dextran chips. In each assay, recombinant FnBPB N23 isotype I was passed over the surface in concentrations ranging from 0.15 \textmu M (lower-most trace) to 10 \textmu M (upper-most trace). The phases of association and dissociation are indicated. The representative sensorgrams have been corrected for the response obtained when recombinant FnBPB proteins were flowed over uncoated chips.
SPR analysis with K

FnBPB isotypes examined in this study bound immobi-

molar range. The K

binding region(s) in human fibronectin. The mechanism for this interaction and identify the FnBPB to examine the biological significance of fibronectin-novel mechanism. Experiments are currently underway to determine fibronectin-binding motif and may bind fibronectin by a novel mechanism. These data have impli-

residues on the surface of the protein and not in regions predicted to be involved in ligand binding. Using the recombinant N23 isotype 1 protein as a prototype, the affinity of FnBPB for fibrinogen and elastin was analysed by SPR. The K

both interactions was in the low micro molar range.

Somewhat surprisingly, the seven recombinant N23 FnBPB isotypes examined in this study bound immobi-

E. coli strain XL-1 Blue (Stratagene). E. coli strain TOPP 3 (Qiagen) was used for the expression of recombinant FnBPB A domain proteins. Ampicillin (100 μg ml

Cloning of fnbB gene fragments

Plasmid DNA was isolated using the Wizard® Plus SV Miniprep kit (Promega) according to manufacturer’s instructions and finally transformed into E. coli XL1-Blue cells using standard procedures [33]. Chromosomal DNA was extracted using the Bacterial Genomic DNA purification kit (Edge Biosystems). Restriction digests and ligation reactions were carried out using enzymes from New England Biolabs and Roche according to the manufacturers’ protocols. Oligonucleotides were purchased from Sigma-Genosys and are listed in Table 3. DNA purification was carried out using the Wizard® SV Gel and PCR Clean-up System (Promega).

DNA hybridisation using fnbB type-specific probes

DIG-labelled isotype-specific probes were synthesised by PCR. Primers were designed to amplify a small region of DNA (~300 bp) in the N3 sub-domain of isotypes I-VII. The PCR products were labelled by incorporating DIG-
### Table 2: *S. aureus* strains screened for FnBPB isotypes.

| Strain | ST | Host | FnBPB | Method of Detection |
|--------|----|------|-------|---------------------|
| 8325-4 | 8  | Human| I     | Genome sequence [9]  |
| N315   | 5  | Human| II    | Genome sequence [38]|
| MSSA476| 1  | Human| III   | Genome sequence [39]|
| P1     | 973| Rabbit| IV    | fnbB gene sequence (Genbank: HM196815) |
| 2      | 7  | Human| V     | fnbB gene sequence (Genbank: HM196814) |
| 19     | 10 | Human| IV    | DNA hybridization    |
| 114    | 39 | Human| II*   | fnbB gene sequence (Genbank: HM196816) |
| 116    | 9  | Human| II    | DNA hybridization    |
| 138    | 30 | Human| II*   | fnbB gene sequence (Genbank: HM196817) |
| 162    | 1  | Human| III   | DNA hybridization    |
| 304    | 39 | Human| II*   | fnbB gene sequence (Genbank: HM196818) |
| 316    | 49 | Human| II    | DNA hybridization    |
| 402    | 13 | Human| IV    | DNA hybridization    |
| 563    | 37 | Human| II*   | fnbB gene sequence (Genbank: HM196819) |
| 617    | 45 | Human| II    | DNA hybridization    |
| 863    | 20 | Human| II    | DNA hybridization    |
| 964    | 18 | Human| II    | DNA hybridization    |
| 3015   | 123| Human| IV    | DNA hybridization    |
| 3077   | 17 | Human| VI    | fnbB gene sequence (Genbank: HM196821) |
| 3084   | 52 | Human| II    | DNA hybridization    |
| 3089   | 97 | Human| IV    | DNA hybridization    |
| 3110   | 12 | Human| V     | fnbB gene sequence (Genbank: HM196820) |
| 3132   | 2  | Human| II    | DNA hybridization    |
| 233    | 45 | Human| VII   | fnbB gene sequence (Genbank: HM196822) |
| PSA5   | 698| Bovine| V    | DNA hybridization    |
| RF79   | 71 | Bovine| IV   | DNA hybridization    |
| MSA1007| 708| Bovine| II   | DNA hybridization    |
| DS37   | 20 | Bovine| II    | DNA hybridization    |
| DS40   | 1  | Bovine| III   | DNA hybridization    |
| DS42   | 479| Bovine| II    | DNA hybridization    |
| MSA915 | 115| Bovine| II    | DNA hybridization    |
| MSA1547| 699| Bovine| IV    | DNA hybridization    |
| MSA1047| 350| Bovine| IV    | DNA hybridization    |
| DS70   | 697| Bovine| II    | DNA hybridization    |
| MSA1363| 25 | Bovine| II    | DNA hybridization    |
| RF26   | 97 | Bovine| III   | DNA hybridization    |
| DS35   | 696| Bovine| IV    | DNA hybridization    |
| MSA1006| 8  | Bovine| I     | DNA hybridization    |
| MSA17.1| 693| Bovine| II    | DNA hybridization    |
| MSA1011| 352| Bovine| II    | DNA hybridization    |
| RF283  | 133| Bovine| IV    | DNA hybridization    |
| MSA1468| 694| Bovine| II    | DNA hybridization    |
| DS36   | 126| Bovine| IV    | DNA hybridization    |
| RF122  | 151| Bovine| absent| Genome sequence [25] |
labelled dNTPs (Roche). Five ng of DNA encoding the A domain of FnBPB from clinical isolates was spotted onto positively charged nylon membranes (Roche) and allowed to air-dry. Membranes were incubated for 5 min on blotting paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH), 5 min in neutralization solution (1.5 M NaCl, 1 M Tris–HCl, pH 7.4), and finally for 15 min on blotting paper soaked with 2× SSC solution (300 mM NaCl, 30 mM tri-sodium citrate). DNA was fixed on the membranes by incubation at 120°C for 30 min. Membranes were incubated for 2 h at 68°C in pre-hybridization solution (5× SSC, 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS and 1× Blocking Reagent (Roche)). DIG-labelled probes were denatured by heating at 95°C for 10 min, diluted in pre-hybridization solution and incubated with nylon membranes for 18 h at 68°C. Following hybridization, the membranes were washed twice with 2× SSC/0.1% w/v SDS at room temperature followed by two washes with 0.5× SSC/0.1% w/v SDS at 68°C for 20 min. Membranes were equilibrated for 30 min in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5), and bound DIG-labelled probes were detected by incubation for 30 min with alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:10,000 in maleic acid buffer. After washing twice with maleic acid buffer containing 0.3% v/v Tween 20, the chemiluminescence substrate CSPD (Roche) was used to detect bound anti-DIG antibodies and membranes were exposed to X-OMAT UV Plus Film (Kodak).

Bioinformatic and phylogenetic analysis of FnBPB A domain isotypes

Protein sequences were aligned in pairwise combinations to calculate amino acid identity using the ExPASy SIM alignment tool http://www.expasy.org/tools/sim-prot.html. The concatenated MLST allele sequences of S. aureus strains were downloaded from the MLST database http://saureus.mlst.net/. A phylogenetic analysis of concatenated MLST allele sequences was conducted using MEGA version 4 [34]. Alignments of multiple protein sequences to view areas of conservation amongst A domains were performed using Clustal W http://www.ebi.ac.uk/

Generation of 3D-models for FnBPB (N23) types I-VII

Theoretical models of the structure of region A (N23) types I-VII were obtained by submitting the amino acid sequences for this segment of each protein to the Phyre service of the 3D-PSSM website http://www.sbg.bioic.ac.uk/phyre/. This web-based tool models the structure of these sequences based structure of the equivalent domains of the S. aureus clumping factor ClfA. All structures were viewed using the pyMOL viewing software.

Expression of recombinant FnBPB A domain proteins

Primers were designed to amplify DNA encoding residues 162-480 (N23 sub-domain) of FnBPB isotype I from strain 8325-4 by PCR. The primers included BamHI and Smal restriction sites to facilitate cloning into the multi-cloning site of the N-terminal six-histidine tag expression vector pQE30 (Qiagen) and incorporated a 3' stop codon. The equivalent N23 regions of FnBPB isotypes types II-VII were PCR-amplified from strains N315, MSSA476, P1, 2, 3077 and 233, respectively. The PCR products were cloned separately into pQE30 and transformed into E. coli cells for protein production. Each construct was verified by sequencing (GATC Biotech AG, Germany) and proteins were purified by Ni2+ chelate chromatography [35]. Concentrations were determined using the BCA Protein Assay Kit (Pierce). Proteins were dialysed against PBS for 24 h at 4°C, aliquoted and stored at -70°C.

Direct binding of recombinant FnBPB A domain proteins to immobilized elastin, fibrinogen and fibronectin

Human aortic elastin (Elastin Products Company; 50 μg/ml) was coated onto microtiter wells for 18 hr under UV light. Wells coated with human fibrinogen (Calbiochem; 10 μg/ml), and fibronectin (Calbiochem; 10 μg/ml) were placed at 4°C overnight. All plates were blocked with 5% skimmed milk in phosphate buffered saline (PBS) for 2 hr at 37°C. Following three washes with PBS containing 0.05% v/v Tween 20 (PBST) various concentrations of purified rFnBPB N23 constructs in PBS were added and incubated at 37°C for 2 hr. After three washes with PBST, bound protein was detected by incubation with a 1:500 dilution of monoclonal antibody 7E8 that recognizes the N-terminal hexahistidine fusion tag. After 1 h incubation with shaking at room temperature, the wells were washed three times with PBST followed by 100 μl per well of goat-anti-mouse IgG antibodies conjugated to horseradish-peroxidase (HRP; Dako; Denmark) diluted 1:2000. After incubation for 1 h at room temperature, wells were washed three times with PBST, and bound HRP-conjugated antibodies were detected with 10 μg per well of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) in 0.05 M phosphate-citrate buffer containing 0.006% (v/v) hydrogen peroxide. After incubation at room temperature for 5 min the reaction was stopped by adding 50 μl of 2 M H2SO4. The absorbance at 450 nm was measured with an ELISA plate reader (Multiskan EX, Labsystems).

The purity of the commercial fibronectin used in these assays was examined by SDS-PAGE. ELISA experiments with anti-fibrinogen antibodies revealed that the fibronectin was free of fibrinogen contamination.
### Table 3: Primers.

#### Flanking primers

| Primer Type | Name | Sequence |
|-------------|------|----------|
| pfnB Adom F | pfnB | CCGGGATCCAGAAAAACACAAATTTGGGAGC |
| pfnB Adom R | pfnB | CCGGGATCCACATGAATAGAATCTTCTTCAG |
| pfnA Adom F | pfnB | CCGAAGCTGTCGAAAAACAAATCTTACAGTAC |
| pfnA Adom R | pfnB | CCGGGATCTATGTAATGATCGATATTCAATC |

#### Type-specific probe primers

| Primer Type | Name | Sequence |
|-------------|------|----------|
| pfnB N3 I F | pfnB | CTGGTCAAGTAACATAAGG |
| pfnB N3 I R | pfnB | GTATAAAATAGTTATAATATC |
| pfnB N3 II F | pfnB | ACTGGTCAGTAACATCTG |
| pfnB N3 II R | pfnB | GTAGTATTTATGATACGATCA |
| pfnB N3 III F | pfnB | TAAAGTGAGAATTGATACG |
| pfnB N3 III R | pfnB | TAATGTAATAACAGTAATAG |
| pfnB N3 IV F | pfnB | ACTGGTCAGTAACATCTG |
| pfnB N3 IV R | pfnB | AGTATAATGTAATAACCTTG |
| pfnB N3 V F | pfnB | CTGGTCAAGTAACATACTGG |
| pfnB N3 V R | pfnB | GAATATAGGTAATAATAGT |
| pfnB N3 VI F | pfnB | GTTAATAGGAAAAACAGTAAG |
| pfnB N3 VI R | pfnB | CTGATATCAGTTAAATACCTTG |
| pfnB N3 VII F | pfnB | ATATAAAACATTTGAGTACAGT |
| pfnB N3 VII R | pfnB | CTCCCACTGAGGCTCAGATTAAATGTC |

#### pQE30 vector primers

| Primer Type | Name | Sequence |
|-------------|------|----------|
| pfnBpQE I F | pfnB | GGGGGATCCCGGTACAGATGTAACAAATAAG |
| pfnBpQE I R | pfnB | AATCCGGGTAATTTGCTTACCATGCA |
| pfnBpQE II F | pfnB | GGGGGATCCCGGTACAGATGTAACAAATAAG |
| pfnBpQE II R | pfnB | GGCCCGGGTTAATTTGCTTACCATGCA |
| pfnBpQE III F | pfnB | CCTGGTACCCGTCAGATGTAACAAATAAG |
| pfnBpQE III R | pfnB | AAATCCCGGTACAGATGTAACAAATAAG |
| pfnBpQE IV F | pfnB | CCTGGTACCCGTCAGATGTAACAAATAAG |
| pfnBpQE IV R | pfnB | ATCCGGGGTAAATTTGCTTACCATGCA |
| pfnBpQE V F | pfnB | TAAATCGGCGTACAGATGTAACAAATAAG |
| pfnBpQE V R | pfnB | ATTCGCCGTTAATTTGCTTACCATGCA |
| pfnBpQE VI F | pfnB | ATGGGGTACCGGTACAGATGTAACAAATAAG |
| pfnBpQE VI R | pfnB | TCTGGGTTAATTTGCTTACCATGCA |
| pfnBpQE VII F | pfnB | CTAGGAGTACCGGTACAGATGTAACAAATAAG |
| pfnBpQE VII R | pfnB | AAATCCCGGGTTTTCCTGCAATCATCC |

* restriction endonuclease sites are italicised.
ELISA assays

Various concentrations of recombinant FnBPB A domain proteins in PBS were coated onto Nunc 96-well microtitre dishes for 18 h at 4°C. Wells were washed and blocked with BSA for 2 h as described above. Following three washes with PBST, 100 μl of anti-FnBPB A domain antibodies diluted in BSA-PBST (1.8 μg polyclonal IgG ml⁻¹; 2.5 μg monoclonal IgG ml⁻¹) were added to each well and incubated for 1 h at room temperature with shaking. Polyclonal antibody raised against the isotype I N23 domain of FnBPB was obtained by immunizing specific pathogen-free rabbits with rFnBPB37-480 from S. aureus 8325-4. Monoclonal antibody 12E11 was generated by immunizing mice with recombinant isotype I FnBPB37-480. After 1 h incubation the wells were washed three times with PBST. Goat anti-rabbit IgG-HRP conjugated antibodies or goat anti-mouse IgG-HRP conjugated antibodies (Dako, Denmark), each diluted 1:2000 in BSA-PBST, were added to the wells and incubated for 1 h. After washing three times with PBST, bound HRP-conjugated antibodies were detected as described above.

Analysis of fibrinogen, elastin and fibronectin binding by surface plasmon resonance

Surface plasmon resonance (SPR) was preformed using the BIAcore x100 system (GE Healthcare). Human fibrinogen (Calbiochem), aortic elastin (Enzyme Research Laboratories) and fibronectin (Calbiochem) were covalently immobilized on CM5 sensor chips using amine coupling. This was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), followed by N-hydroxysuccinimide (NHS) and ethanolamine hydrochloride, as described by the manufacturer. Fibrinogen (50 μg/ml), elastin (50 μg/ml) and fibronectin (50 μg/ml) were dissolved in 10 mM sodium acetate at pH 4.5 and immobilized on separate chips at a flow rate of 30 μl/min in PBS (Gibco). Each chip contained a second flow cell, which was uncoated to provide negative controls. All sensorgram data presented were subtracted from the corresponding data from the blank cell. The response generated from injection of buffer over the chip was also subtracted from all sensorgrams. Equilibrium dissociation constants (Kd) were calculated using the BIA x100 evaluation software version 1.0.

Authors' contributions

FMB carried out cloning of fnbA genes for sequencing and protein expression, DNA and amino acid sequence analysis, fnbA DNA hybridization experiments, phylogenetic analysis, purification of recombinant A domain proteins, ELISA experiments, SPR experiments and drafted the manuscript. NMC carried out fnbB DNA hybridization experiments involving bovine S. aureus strains; PS and SR were responsible for production of polyclonal and monoclonal antibodies against the isotype I A domain. TJF conceived and coordinated the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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