A second IgG-binding protein in *Staphylococcus aureus*

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Most strains of *Staphylococcus aureus* express IgG-binding activity and this binding has been considered to be solely mediated by protein A. However, the existence of a second gene in *S. aureus* strain 8325-4 encoding an IgG-binding polypeptide was recently reported. This novel IgG-binding polypeptide was found after panning a shotgun phage display library, made from chromosomal DNA, against immobilized human IgG. The complete gene (*sbi*) encoding this novel IgG-binding protein, denoted protein Sbi, has now been cloned and sequenced. Analysis of other *S. aureus* strains showed that this gene is not unique for strain 8325-4. The protein consists of 436 amino acids and exhibits an immunoglobulin-binding specificity similar to protein A. Furthermore, it is shown that Sbi is highly expressed in strain Newman 4, which shows that IgG-binding activity in *S. aureus* can be mediated by proteins other than protein A.

Keywords: *Staphylococcus aureus*, IgG-binding protein, Sbi and phage display

INTRODUCTION

*Staphylococcus aureus* is a pathogen responsible for a wide variety of diseases in humans and animals, including endocarditis, osteomyelitis, wound sepsis and mastitis (Easmon & Adlam, 1983). The bacterium produces several potential virulence factors such as alpha-, beta-, gamma- and delta-toxins, toxic shock syndrome toxin, enterotoxins, leucocidin, proteases, coagulase and clumping factor (Iandolo, 1989). It is generally accepted that adhesion to tissues is required for bacterial colonization to occur. For this purpose, staphylococci express surface adhesins which interact with host matrix proteins such as fibronectin, vitronectin, collagen, laminin and bone sialoprotein (Patti et al., 1994). In addition, staphylococci are able to bind several serum proteins, such as IgG, fibronectin, fibrinogen and thrombospondin, possibly masking the bacteria from the immune system of the host (Patti et al., 1994). However, the contribution and importance of each of these binding functions in different infections is still unclear.

The most studied receptor in *S. aureus* is protein A, a cell-wall-associated protein which binds to the Fc and the Fab regions of IgG from several species. Protein A in strain 8325-4 consists of five consecutive highly homologous domains, all with IgG-binding activity, followed by a region anchoring the protein in the cell wall (Forsgren & Sjoquist, 1970; Forsgren et al., 1971). IgG-binding ability is common among clinical strains of *S. aureus* (Forsgren & Forsum, 1970; Forsgren et al., 1971), suggesting an important function in pathogenesis. The IgG-binding capacity has been thought to be mediated by protein A only. However, we recently identified a nucleotide sequence in *S. aureus* strain 8325-4 encoding a polypeptide, clearly distinguishable from protein A, which binds IgG in a non-immune fashion (Jacobsson & Frykberg, 1995). We now report on cloning and nucleotide sequence determination of the gene encoding this novel IgG-binding protein. The gene encodes a protein of 436 amino acids with one functional IgG-binding domain and without the typical Gram-positive cell wall anchoring sequence LPXTG (Schneewind et al., 1992, 1995; Navarre & Schneewind, 1994), suggesting that the protein is not anchored in the cell wall. This gene is present in all tested strains of *S. aureus*.

METHODS

Bacterial strains, growth conditions, vectors and helper phage. The bacterial strains used are listed in Table 1. Phage R408 (Promega) was used as the helper phage for production
Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Characteristics and use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>TG1</td>
<td>F+ and amber suppressing; used for construction of the phage library and production of phage stocks</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>MC1061</td>
<td>Used for all other DNA manipulations</td>
<td>Wertman et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>8325-4</td>
<td>NCTC 8325 cured from prophages</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td></td>
<td>Wood 46</td>
<td>Protein-A-negative reference strain</td>
<td>Kronvall et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>Newman 4</td>
<td>Spontaneous mutant of strain Newman with enhanced production of fibronectin-binding protein</td>
<td>Jönsson et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Cowan I</td>
<td>NCTC 8350, high level producer of cell-wall-bound protein A</td>
<td>Forsgren et al. (1971)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>247</td>
<td></td>
<td>Rosendorf &amp; Kayser (1974)</td>
</tr>
</tbody>
</table>

Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Use</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pe</td>
<td>Sequencing of pHEN1 clones</td>
<td>5’-TTG CCT ACG GCA GCC GCT GAA-3’</td>
</tr>
<tr>
<td>My</td>
<td>Sequencing of pHEN1 clones</td>
<td>5’-TGC GGC CCC ATT CAG ATC CTC-3’</td>
</tr>
<tr>
<td>Olg1</td>
<td>Sequencing of sbi</td>
<td>5’-CTC CAT ATA GTA CTT CCT TA-3’</td>
</tr>
<tr>
<td>Olg2</td>
<td>Sequencing of sbi</td>
<td>5’-GAG ATT GCA TCA TTT TGT TTT GAT-3’</td>
</tr>
<tr>
<td>Olg3</td>
<td>Sequencing of sbi</td>
<td>5’-GTA ACC ATA GTT AAA TGA AT-3’</td>
</tr>
<tr>
<td>Olg4</td>
<td>Sequencing of sbi</td>
<td>5’-CGA TAA ATC AGC AGC ATA TG-3’</td>
</tr>
<tr>
<td>Olg5</td>
<td>Sequencing of sbi</td>
<td>5’-CAA TCA CCA CAA ATT GAA AA-3’</td>
</tr>
<tr>
<td>Olg6</td>
<td>Sequencing of sbi</td>
<td>5’-TGG TGC TTG TAG TGG AAA AG-3’</td>
</tr>
<tr>
<td>Olg8</td>
<td>PCR for MAL–Sbi fusions</td>
<td>5’-AGT GGA TCC ACG CAA CAA ACT TCA ACT AAG CA-3’</td>
</tr>
<tr>
<td>Olg9</td>
<td>PCR for MAL–Sbi fusions and construction of sbi probe</td>
<td>5’-AATGTCGACAAACTAGAGAAGATA AAG A-3’</td>
</tr>
<tr>
<td>Olg10</td>
<td>PCR for MAL–Sbi fusions and construction of sbi probe</td>
<td>5’-TAG GAT CCG TAC AAT CTT CTA AAG</td>
</tr>
</tbody>
</table>

of phage stocks. *Escherichia coli* containing the pUC18 or pMAL-c2 vectors (New England Biolabs) was selected on LA plates (Luria–Bertani (LB) broth with 1.5% agar and 50 µg ampicillin ml\(^{-1}\)) and grown in LB broth supplied with 50 µg ampicillin ml\(^{-1}\). *E. coli* containing the phagemid vector pHEN1 (Hoogenboom et al., 1991) was grown in the same medium supplemented with 1% (w/v) glucose. Staphylococcal strains were grown in Tryptone Soya Broth (Oxoid). *Staphylococcus epidermidis* containing pRB473 (shuttle vector kindly provided by R. Brückner, Tübingen, Germany) constructs was grown in the same medium containing 20 µg chloramphenicol ml\(^{-1}\).

Cloning and DNA sequencing. Restriction and modification enzymes were purchased from Promega, Amersham International or Boehringer Mannheim. Oligonucleotides were synthesized by Scandinavian Gene Synthesis or Pharmacia Biotech and are listed in Table 2.

All DNA manipulations were performed using standard methods (Sambrook et al., 1989), except ligations and small-scale plasmid preparations, for which the Ready to Go ligation kit (Pharmacia Biotech) and Wizard Minipreps DNA Purification Systems (Promega), respectively, were used according to the manufacturers’ instructions. Plasmids were introduced into *E. coli* and staphylococci by electrottransformation (Oskouin & Stewart, 1990). Staphylococcal chromosomal DNA was prepared according to Lindberg et al. (1972).

DNA was sequenced according to the dideoxy chain-termination method using the Sequenase version 2.0 DNA Sequencing kit from United States Biochemical. Restriction sites shown in Fig. 1 were used for construction of subclones used in determination of the nucleotide sequence. One additional clone, pHSBB7, was made (not shown) by Bal31 exonuclease digestion from the unique HindIII site in the 5’ direction of the gene, and sequenced. Different oligonucleotides were used as primers for determining the sequence of both DNA strands (Table 2). The PC/GENE program (IntelliGenetics) was used for the handling of the sequences. The EMBL, GenBank, SWISS-PROT and PIR databases were searched for sequence homologies.

To express the Sbi protein, two constructs were made in the pMAL-c2 vector. Primers Olg8 and Olg9 were used to PCR-amplify the DNA encoding full-length protein lacking the signal sequence (aa 33–436). Primers Olg9 and Olg10 were used to PCR-amplify the DNA encoding a truncated version.
(aa 143–436) lacking also the IgG-binding domain. The obtained PCR products were digested with the restriction enzymes BamH1 and SalI and ligated into the vector cleaved with the same enzymes and transformed into E. coli.

Southern blots and hybridizations were performed according to Sambrook et al. (1989), using a Ncol–Xhol fragment, i.e. the complete insert from clone ig4 (Jacobsson & Frykberg, 1995) 32P-labelled by random priming for detection and cloning of the sbi gene. For detection of the sbi gene in different S. aureus strains, the PCR fragment obtained by using olig9 and olig10 (see above) was 32P-labelled by random priming. The hybridization was carried out at 50 °C and the washing at 65 °C in 0·1× SSC (20× SSC = 3·0 M NaCl and 0·3 M sodium citrate) and 0·1% SDS.

Shotgun mapping by phage display. The library was constructed from the cloned sbi gene essentially as described by Jacobsson & Frykberg (1995). In short, the DNA from clone pPX1 (Fig. 1a) was sonicated and DNA fragments of approximately 50–300 bp were isolated by preparative gel electrophoresis. The fragments were blunt-ended with T4 DNA polymerase and ligated into the phagemid pHEN1, previously digested with PstI, blunt-ended, and dephosphorylated with calf intestine alkaline phosphatase. The ligation was made using 1 μg vector and 1 μg DNA fragments and the mixture was transformed into E. coli TG1. The transformants were grown overnight in LB supplied with 50 μg ampicillin ml−1 and 1% (w/v) glucose and thereafter infected with helper phage R408 at an m.o.i. of 20. After 1 h, the culture was diluted and ampicillin was added to a final concentration of 50 μg ml−1. After 5 h growth at 37 °C, the bacteria were pelleted and the supernatant, containing the phages, was sterilely filtered.

The library was affinity-selected against human IgG (KabiVitrum) and positive clones were identified using labelled IgG as described by Jacobsson & Frykberg (1995).

Determination of the specificities of Sbi and protein A. To determine the specificity of Sbi, a phage stock was prepared as described above from clone Ig4, encoding the IgG-binding domain of Sbi (Jacobsson & Frykberg, 1995). The stock was diluted to 105 phagemid particles ml−1 and 100 μl was plated as described by Jacobsson & Frykberg (1995) against human IgG, IgM, IgA, IgG3a and IgG3i as well as IgG from rat, goat, pig, cow, sheep, horse, guinea pig, dog, rabbit and chicken (Sigma), immobilized in microwells at a concentration of 50 μg ml−1. BSA was included as a negative control. The number of bound phagemid particles was determined as c.f.u.

Protein purification and electrophoresis. The MAL fusion proteins MAL–Sbi (aa 33–346) and MAL–SbiA (aa 143–436) were purified from E. coli lysates on an amylene resin according to the manufacturer’s instructions (New England Biolabs). MAL–Sbi was further purified on IgG–Sepharose according to the manufacturer’s instructions (Pharmacia Biotech).

Antibodies against Sbi, developed in chickens, were obtained through Immunosystem (four immunizations of 50 μg MAL–SbiA protein). The antibodies were affinity-purified on immobilized MAL–SbiA protein and labelled with horseradish peroxidase (HRP; Boehringer Mannheim). Commercially available HRP-labelled chicken antibodies against protein A were obtained from Immunosystem.

The bacterial cells from overnight cultures of Newman 4 (2 ml), Cowan I (2 ml) and S. epidermidis containing pshPX1 (40 ml) were collected by centrifugation and washed once in PBS-D (140 mM NaCl, 8 mM Na2HPO4, 1·7 mM KH2PO4, 2·7 mM KCl; pH 7·4). Newman 4 and S. epidermidis cells were resuspended in 40 μl PBS, followed by addition of 40 μl 2× sample buffer (1× buffer = 62·5 mM Tris/HCl, pH 6·8, 10% glycerol, 5% v/v, β-mercaptoethanol, 2% SDS and 0·01% bromophenol blue) and the samples were boiled for 2 min. To release protein A, the same amount of cells in PBS was treated with lysostaphin (0·1 mg ml−1; Sigma) for 5 min at 37 °C before addition of the sample buffer and boiling for 2 min. Samples (1 μl) were analysed by SDS-PAGE using the Phast system (Pharmacia Biotech) with PhastGel Gradient 8–25% gels and PhastGel SDS Buffer Strips. Also included were 0·025 μg protein A (Pharmacia Biotech), 0·05 μg MAL–Sbi and 0·05 μg MAL–SbiA. The proteins were blotted onto nitrocellulose filters (Schleicher & Schuell) and proteins were detected using HRP-labelled affinity-purified rabbit anti-chicken (diluted 1/500; Sigma), anti-Sbi (10 μg ml−1) or anti-protein A (15 μg ml−1) antibodies. Bound antibodies were detected using 4-chloro-1-naphthol (Serva).

RESULTS

Cloning and sequencing of the sbi gene encoding an IgG-binding protein

The original clone (Fig. 1b, clone Ig4) expressing an IgG-binding polypeptide was earlier isolated from a shotgun phage display library made from S. aureus strain 8325-4 (Jacobsson & Frykberg, 1995). The insert from this clone was used as a probe for identification and subsequently for cloning of the complete gene from S. aureus strain 8325-4. Chromosomal DNA was digested with PstI and XbaI, and the DNA fragments were separated by agarose gel electrophoresis followed by blotting onto a nitrocellulose filter. Hybridization with the Ncol–Xhol fragment, derived from Ig4, showed that the gene resided on a fragment of approximately 3 kb in size. DNA fragments of this size were purified by agarose gel electrophoresis and cloned into the pUC18 vector.

One clone, pPX1, hybridizing with the probe was further characterized by restriction enzyme analysis. Fig. 1 schematically shows the 3 kb PstI–XbaI DNA fragment containing the sbi gene and the different restriction enzymes used for subcloning.

The nucleotide sequence (GenBank accession number AF027153) encodes a protein of 436 amino acids, including a typical signal peptide with a putative cleavage site after amino acid 29. The gene has the normal features associated with a functional gene: putative promoter sequences, a ribosome-binding site and an inverted repeat located after the translation termination stop codon. Only a part of the encoded protein shows homology to protein A, while the rest of the protein does not show any homology to any known protein. A proline-rich sequence, containing eight prolines repeated every fifth amino acid, starts at position 267. Such sequences are normally found within cell-wall-spanning domains. However, in this case the proline-rich region is not followed by the cell-wall-sorting LPXTG motif (Schneewind et al., 1992, 1995; Navarré & Schneewind, 1994).
Mapping of the IgG-binding domain in Sbi

To determine the exact position of the IgG-binding domain in Sbi, a shotgun phage display library was made from clone pPX1. After panning the library against immobilized human IgG, E. coli TG1 cells were infected with the eluted phage and the bacteria were spread on LA plates containing ampicillin. AmpR colonies were analysed for binding of HRP-labelled human IgG. Positive clones were isolated and the nucleotide sequences of the different inserts were determined. As seen in Fig. 1, all clones had inserts derived from the same region. 194 represents the clone isolated previously (Jacobsson & Frykberg, 1995). S denotes the signal sequence and the hatched bar represents a proline-rich region.

Fig. 1. (a) Cloned 3 kb PstI-XbaI fragment from S. aureus strain 8325-4 containing the sbi gene (clone pPX1). (b) Schematic drawing of Sbi aligned with the peptides encoded by the phagemid clones isolated by panning against IgG. Asterisks indicate that the clone was isolated several times independently. Ig4 represents the clone isolated previously (Jacobsson & Frykberg, 1995).

Analysis of the protein encoded by the sbi gene

To further characterize the protein encoded by the sbi gene, two clones were made in the pMAL-c2 vector, expressing the mature full-length protein (MAL–Sbi; aa 33–436) and a truncated protein (MAL–SbiA; aa 143–436) lacking the IgG-binding domain. The purified products were analysed by SDS-PAGE and Western blotting together with commercially available protein A, cell-surface extract of S. epidermis containing pShX1 and S. aureus Newman 4, as well as lysed Newman 4 and Cowan I cells. Three duplicate gels were blotted onto nitrocellulose filters and the blots were developed with HRP-labelled rabbit IgG, HRP-labelled anti-protein A and HRP-labelled anti-Sbi, respectively (Fig. 4a–c). Fig. 4(a) shows that the MAL fusion of full-length protein Sbi and Sbi expressed in S. epidermis bind IgG, while MAL–SbiA, lacking the first 110 aa, does not bind IgG. IgG-binding activity is also detected in Newman 4 and Cowan I. As shown in Fig. 4(b), commercially available anti-protein A antibodies developed in chickens react with full-length Sbi and Sbi expressed in S. epidermis bind IgG, while MAL–SbiA, lacking the first 110 aa, does not bind IgG.

Analysis of the immunoglobulin specificity of Sbi

To analyse the immunoglobulin-binding properties of Sbi, clone Ig4 encoding the IgG-binding domain was used to produce phage particles displaying the binding domain on the surface. These phages were panned against different immunoglobulins and the number of binding phagemid particles was determined and used as a measure of the binding ability. As shown in Fig. 3, human, pig, rabbit and guinea pig IgG are bound most strongly.

Fig. 2. Alignment of the deduced minimal IgG-binding domain of Sbi (SBIM; aa 41–92) with the repeated IgG-binding domains A–E of staphylococcal protein A (Uhlen et al., 1984). Asterisks indicate amino acids present at the same position in all repeats of protein A and in Sbi. Dots represent amino acids present in at least one of the repeats of protein A and in Sbi. Shown in bold are residues in the B domain of protein A implicated in contributing to the contact with C2–C3 of the IgG molecule (Deisenhofer, 1981).
Occurrence of the sbi gene in staphylococcal strains

Chromosomal DNA was isolated from S. aureus strains 8325-4, Cowan I, Newman 4 and Wood 46. The DNAs were digested with HindIII followed by agarose gel electrophoresis and then blotted onto a nitrocellulose filter. The sbi gene was detected in all four S. aureus strains tested (Fig. 5).

DISCUSSION

Analysis of IgG-binding activity in S. aureus by Western blotting usually reveals more than one protein band that interacts with IgG, and it has been assumed that these polypeptides represent breakdown products of protein A. As shown in this study, a second IgG-binding protein, Sbi, is expressed in S. aureus. However, it is not surprising that Sbi has escaped detection as analysis of the expression of Sbi in S. aureus has been hampered by the ability of protein A to interact with IgGs from most mammalian species. Furthermore, both proteins migrate similarly in SDS-PAGE and have the same specificity for all tested immunoglobulins. As shown in Fig. 4(b), the commercially available anti-protein A antibodies cross-react with full-length Sbi but not with MAL-SbiA (lacking the IgG-binding domain). It is likely that the cross-reaction is not unique to this source of antibodies and may have contributed to the failure to identify Sbi. Now the antibodies directed against Sbi produced in chickens allow discrimination between the expression of protein A and Sbi.

We have cloned and sequenced the sbi gene from S. aureus strain 8325-4. The detection of Sbi, expressed from its own promoter in S. epidermidis and as a MAL fusion in E. coli with HRP-labelled IgG, proves that this gene encodes a second IgG-binding protein. This protein consists of 436 amino acids and contains a signal sequence but lacks the cell-wall-sorting LPXTG motif, suggesting that the protein is not anchored in the cell wall. However, in strain Newman 4, which produces
high amounts of Sbi, no Sbi is detected in the culture growth medium (data not shown). Instead, Sbi is released from the cell surface by addition of sample buffer (Fig. 4). In contrast, release of protein A requires treatment with lysostaphin (Fig. 4). This indicates that Sbi is associated with the cell surface by a different mechanism. The predicted pKₐ of mature Sbi is 9.8, which makes the protein positively charged at a neutral pH. Perhaps the association between the bacteria and the Sbi protein is partly electrostatic since the protein is also released by addition of SDS (data not shown). There are also other examples of cell-surface-associated proteins that lack the LPXTG motif, such as the S. aureus elastin-binding protein (Park et al., 1996) and major histocompatibility complex class II analogue (Jonsson et al., 1995).

Mapping of Sbi by shotgun phage display suggests that the protein has one functional IgG-binding domain with a deduced minimal binding domain of 52 amino acids. Furthermore, expression and analysis of NH₂-terminally truncated Sbi consisting of aa 143–436 show that no IgG-binding activity is located in this part of the protein (Fig. 4). The IgG-binding domain shows significant homology to the IgG-binding repeats of protein A (Fig. 2). Interestingly, the highest homologies are found in the regions involved in the binding of the B domain of protein A to the Fc fragment of IgG (Deisenhofer, 1981).

Of the 11 amino acids in the B domain involved in the binding of IgG, seven are identical, one is conservatively changed and three are substituted in Sbi. Analysis of the deduced amino acid sequence suggests the presence of a second IgG-binding domain immediately downstream of SbiM. However, no clones expressing this region were recovered in the mapping experiment. Still, it cannot be excluded that this region constitutes a second IgG-binding domain or at least contributes to the IgG-binding activity of protein Sbi.

A phage stock made from the originally isolated clone Ig4 (aa 38–121) (Fig. 1b) was used in an analysis of the immunoglobulin species reactivity (Fig. 3). A comparison between data on the specificity of protein A (Boyle, 1990) and the results for clone Ig4 (Fig. 3) shows that the two proteins exhibit a very similar immunoglobulin-binding profile. In addition, a study using one or two binding domains from protein A [clones Ig7 (domain C) and Ig1 (D–A) described by Jacobsson & Frykberg, 1995] gave an immunoglobulin-binding profile similar to that of Ig4 (data not shown). The display of protein domain(s) on the phage surface offers a quick and sensitive method for analysing specific binding to other molecules.

It is interesting to note that there are regions of homology between gene sbi and one of the S. aureus genes encoding a fibrinogen-binding protein (fib) (Boden & Flock, 1994). The homology is found in two regions: 5' of the translational start codon, nucleotide positions 86–108 (22/23 nt); and over the translational start codon, nucleotide positions 179–193 (15/15 nt). This might indicate a common regulation of the expression of these two genes. The homology between the genes spa and sbi is limited to the domain(s) encoding IgG-binding activity and no homology is found within the promoter sequences, suggesting that the genes are regulated in different ways.

The ability to bind IgG is very common among staphylococci and streptococci, which implies that IgG binding is of importance in pathogenesis. However, mutants low in expression of protein A and mutants with an inactivated protein A gene are only slightly less virulent than the wild-type strains (Jonsson et al., 1983; Patel et al., 1987). The existence of a second gene encoding an IgG-binding protein in such mutants may explain the failure to show that IgG binding indeed is of importance in virulence. The results in Fig. 5 show that the sbi gene is not unique to strain 8325-4. The fact that Sbi has not earlier been reported in protein-A-negative mutants suggests that the expression level in these strains is low, at least in bacteria grown in vitro. This is supported by the findings that high levels of Sbi were detected in strain Newman 4, whilst it was almost undetectable in strain 8325-4 (data not shown) and not at all detectable in strain Cowan I (Fig. 4c). Thus, a mutant lacking both genes encoding IgG-binding proteins will be required for settling the importance of IgG binding in the virulence of S. aureus.

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