A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family

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Staphylococcus aureus bacteria, isolated from bone and joint infections, specifically interact with bone sialoprotein (BSP), a glycoprotein of bone and dentine extracellular matrix, via a cell-surface protein of M_r 97000 [Yacoub, Lindahl, Rubin, Wendel, Heinegård and Rydén, (1994) Eur. J. Biochem. **222**, 919–925]. Amino acid sequences of seven trypsin fragments from the 97000-M_r BSP-binding protein were determined. A gene encoding a protein encompassing all seven peptide sequences was identified from chromosomal DNA isolated from *S. aureus* strain O24. This gene encodes a protein with 1171 amino acids, called BSP-binding protein (Bbp), which displays similarity to recently described proteins of the Sdr family from *S. aureus*. SdrC, SdrD and SdrE encode putative cell-surface proteins with no described ligand specificity. Bbp also shows similarity to a fibrinogen-binding protein from *S. epidermidis* called Fbe. A serine–aspartic acid repeat sequence was found close to the cell-wall-anchoring Leu-Pro-Xaa-Thr-Gly sequence in the C-terminal end of the protein. *Escherichia coli* cells were transformed with an expression vector containing a major part of the *bbp* gene fused to the gene for glutathione S-transferase. The affinity-purified fusion protein bound radiolabelled native BSP, and inhibited the binding of radiolabelled BSP to staphylococcal cells. Serum from patients suffering from bone and joint infection contained antibodies that reacted with the fusion protein of the BSP-binding protein, indicating that the protein is expressed during an infection and is immunogenic. The *S. aureus* Bbp protein may be important in the localization of bacteria to bone tissue, and thus might be of relevance in the pathogenicity of osteomyelitis.

Key words: adhesion, bone, extracellular matrix, osteomyelitis, serology.

INTRODUCTION

Staphylococcus aureus is the most common pathogen isolated from patients suffering from osteomyelitis and septic arthritis [1,2]. Unless in association with trauma or surgery to the skeleton, these infections occur mainly in young individuals [2] who have a high turnover of bone components [3]. We have previously reported on the specific interaction between bone sialoprotein (BSP), an extracellular matrix (ECM) glycoprotein of M_r 59000 Da, found only in bone and dentine [3–5], and *S. aureus* cells [6,7]. This interaction is potentially of relevance for the understanding of pathogenetic mechanisms in osteomyelitis. It is based on the following facts: (i) BSP is selectively bound by *S. aureus* strains isolated from infections of bone and joint tissue [6,8]; and (ii) particularly high concentrations of BSP are found in the osteoid, the newly formed bone tissue of growing bone, which is the most common site for osteomyelitis [9].

The ability of staphylococcal cells to adhere to ECM components is believed to be important during the early steps of the infectious process. The adhesive molecules present on the cell surface of *S. aureus* include the collagen adhesin (Cna) [10], fibronectin-binding proteins A and B (FnBPA and FnBPB) [11], the clumping factors A and B (ClfA and ClfB), which bind fibrinogen [12,13], an elastin-binding protein (EbpS) [14], and proteins with a broad binding activity [15]. Adhesion of staphylococci to cartilage is mediated by Cna [16,17]; adhesion of the staphylococci to foreign material, such as indwelling devices, depends on ClfA and ClfB, as well as on FnBPA and FnBPB [18,19]. These ECM-binding staphylococcal proteins are involved in the pathogenicity of staphylococci. FnBPA and FnBPB are involved in foreign body infection due to fibronectin-containing deposits being laid down on such material when present in body compartments [18,19]. Furthermore, it has been suggested that FnBPA and FnBPB assume a role in the pathogenesis of endocarditis [20,21], although this finding has been disputed [22]. It has been found that fibrinogen and fibrin are also deposited on indwelling devices, as well as being present in thrombi. ClfA and ClfB mediate attachment of staphylococcal cells to these substrates [18,23], although the pathophysiological relevance of these adhesion mechanisms is not fully clarified. The Cna is a virulence factor in septic arthritis [24], although a Cna-negative isogenic mutant strain was still able to induce septic arthritis in 30 % of infected mice, compared with 70 % for the wild-type staphylococcal strain [24]. These data indicate that several adhesive mechanisms are probably involved in the infectious process of staphylococci.

Structural similarities between FnBPA, Cna, ClfA and Protein A have been reported, as reviewed by Foster and Höök [25]. Structurally, these proteins possess a signal sequence in their N-terminal ends, followed by a series of domains, of which some are

Abbreviations: BSP, bone sialoprotein; Bbp, BSP-binding protein; CIP, calf-intestinal alkaline phosphatase; ClfA/B, clumping factors A and B respectively; Cna, collagen adhesin; ECM, extracellular matrix; FnBPA/B, fibronectin-binding proteins A and B respectively; GST, glutathione S-transferase; SD repeat, serine–aspartic acid repeat; TBS, Tris-buffered saline; TSB, Trypticase soya broth.

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The nucleotide sequence for *bbp* reported in this paper appears in the GenBank Nucleotide Sequence database under the accession number Y18653.

repetitive and mediate the binding to host components. The C-terminal part of these proteins has several characteristic features involved in the anchoring of the respective protein to the bacterial cell wall [26]. The fibrinogen-binding proteins ClfA and ClfB differ from the Cna, FnBPA and FnBPB in that the former pair contain an additional repetitive R-region composed of Ser–Asp (SD) dipeptide repeats [13,25,27–30]. Recently, three additional *S. aureus* genes encoding putative cell-wall proteins containing SD-repeat regions were identified [31]. The distinct N-terminal A-domains of these latter proteins, SdrC, SdrD, and SdrE, were suggested to bind to different ligands, although no specific ligands were identified. A fibrinogen-binding protein from *S. epidermidis*, named Fbe, shows structural similarities to the Sdr proteins [25,32].

BSP comprises approximately 10 % of the non-collagenous proteins of the organic matrix of bone, and contains 40–50 % carbohydrate with a high content (15 %) of sialic acid [4,33]. BSP has an Arg-Gly-Asp (RGD) sequence in the C-terminal end, which confers mammalian cell-binding properties to the protein [5,34]. Staphylococcal binding of BSP depends critically on a Leu-Lys-Arg-Phe (LKRF) sequence located in the N-terminal part of BSP [35]. A 97000-M_r BSP-binding protein from LiCl-extracted cells of the *S. aureus* strain O24 has been isolated previously [36]. In the present study we describe the molecular cloning of the staphylococcal gene encoding this protein.

MATERIALS AND METHODS

Reagents

The enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Xba*I, *Not*I, *Hin*cII, calf intestinal alkaline phosphatase (CIP), and *Taq* DNA polymerase were purchased from MBI (MBI Fermentas Ltd., Vilnius, Lithuania). The 'Ready to go' ligation kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Qiagen Plasmid Kit was from Qiagen (Hilden, Germany). Ovalbumin grade V, BSA fraction V, ampicillin, glutathione and Protein A–alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glutathione S-transferase (GST) and Percoll were purchased from Amersham Pharmacia Biotech. Bovine BSP was kindly provided by Professor Dick Heinegård, (Department of Cell and Molecular Biology, University of Lund, Sweden). Teichoic acid and α-toxin were purchased from BioSys Inova (Stockholm, Sweden). Trypticase soya broth (TSB), yeast extract and agar-base were purchased from Oxoid Ltd., Basingstoke, U.K. ^{125}I-labelled dCTP and [^{32}P]dCTP were purchased from Amersham (Little Chalfont, Bucks., U.K.).

Bacterial strains and plasmids

Escherichia coli strain DH5α was used as the bacterial host for DNA transformation and was grown at 37 °C overnight in Luria Broth (LB; Difco Laboratories, Chicago, IL, USA) supplemented with ampicillin (50 μg/ml). The plasmid vector pBluescript II SK (+) (Stratagene, La Jolla, CA, U.S.A.) was used for subcloning. Chromosomal DNA was prepared from *S. aureus* O24, a strain isolated from a patient suffering from acute osteomyelitis, which was subcultured on blood agar and grown in TSB medium at 37 °C overnight. *E. coli* pGB1 expressing a GST fusion protein were grown in 2 × YTA medium (15 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 100 μg/ml ampicillin) at 37 °C overnight.

Isolation of internal fragments and amino acid sequence analysis

Cells of *S. aureus* strain O24 were grown overnight in TSB, harvested by centrifugation and washed three times in PBS. The cells were treated with 1.0 M LiCl, pH 5.0, to extract cell-surface components. After centrifugation at 10000 ***g*** the supernatant was dialysed against NaCl and P_i. LiCl extracts were passed through IgG–Sepharose and Mono-S columns to avoid interactions with either IgG-binding proteins or a multifunctional cell-surface protein found on staphylococci with affinity for the cation exchanger [15]. The material was subjected to ion-exchange chromatography on a Mono-Q column attached to an FPLC system (Amersham Pharmacia Biotech), from which retained material was eluted at 0.6–0.8 M NaCl. Eluted material was dialysed against buffer B3 (137 mM NaCl, 0.7 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM Hepes, pH 7.4), followed by affinity chromatography on a BSP–Sepharose column that was eluted with a low-pH buffer (0.1 M glycine, pH 3.0), and finally separated by SDS/PAGE [36]. The BSP-binding, Coomassie-Blue-stained band of M_r 97000 was excised and subjected to in-gel digestion [37]. Briefly, the gel slice was washed with NH_4HCO_3 buffer containing 50 % (v/v) acetonitrile before drying. Modified trypsin, sequence grade (Promega, Madison, WI, U.S.A.) was given sufficient time to absorb into the gel; after addition of ammonium bicarbonate buffer, the sample was incubated overnight at 30 °C. Generated peptides were recovered from the supernatant and combined with extracts from the gel slice. Peptides were isolated on a narrow-bore, reversed-phase column (μRPC C2/C18 SC 2.1/100) operated in the SMART system (Amersham Pharmacia Biotech). Seven peptides were sequence-analysed in a Procise 494A automated sequencer (PE Biosystems, Foster City, CA, U.S.A.), operated according to the manufacturer's instructions.

Oligonucleotides

Oligonucleotides were either synthesized in a Gene Assembler Plus instrument (Amersham Pharmacia Biotech) by following the standard protocols, or ordered from Amersham Pharmacia Biotech or DNA Technology (Aarhus, Denmark).

Identification of the gene encoding BSP-binding protein

Two degenerate oligonucleotides, 5′-TAYGAYAARGARACI-ATHCC-3′(*obp1*) and 5′-TCRTCYTCIGCICCIGG-3′ (*obp2*), were designed from two different peptide sequences, YDKETIP (corresponding to amino acids 326–332, as shown in Figure 1) and PGAEDD (corresponding to amino acids 551–556 in Figure 1), obtained from peptide fragments isolated and sequenced from purified BSP-binding protein from *S. aureus* O24. Chromosomal DNA from *S. aureus* strain O24 was used as DNA template for PCR. The reaction was performed in a buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 125 μM dNTPs; 4.6 μM of each degenerate primer and 1.5 units of AmpliTaq DNA polymerase were added in a total volume of 50 μl. The PCR program selected was as follows: 1 min at 95 °C, 1 min at 50 °C, 2 min at 72 °C for 35 cycles, and 7 min at 72 °C. The PCR products were subcloned into a T vector [38] that was based on pBluescript II KS (+). One of the clones harbouring an approx. 700-bp insert was chosen for further studies (pBBP1; see Figure 2). The DNA sequences of the inserts of the various clones obtained were analysed by employing the method of primer walking.

Southern blot analysis and isolation of the complete *bbp* gene

Chromosomal DNA from *S. aureus* strain O24 digested with *Eco*RI was electrophoresed on a 1 % (w/v) agarose gel and transferred to a nylon membrane (Hybond-N$^+$; Amersham, U.K.). The insert of pBBP1, labelled with [^{32}P]dCTP using the

Megaprime labelling kit (Amersham, U.K.), was used to hybridize immobilized DNA at 42 °C overnight; the filter was then washed with 0.1 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 0.1 % (w/v) SDS at 65 °C, and then analysed by autoradiography. A mini-genomic library was constructed by subcloning approx. 4-kb O24 genomic fragments digested with *Eco*RI and subcloned into pBluescript II KS (+) (results not shown). This library was colony-hybridized using the approx. 700-bp insert of pBBP1 as a probe resulting in the isolation of pBBP3 (see Figure 2). Upon further analysis using the same probe, the central part of the *bbp* gene was subcloned into an approx. 1.2-kb *Hin*cII fragment, generating the clone pBBP2 (see Figure 2). To obtain the 3′-end of the gene, additional Southern blot analysis was performed in which the chromosomal DNA was digested with various enzymes. Using the insert of pBBP2 as a probe, the end of the gene was found to be located on an approx. 4-kb *Eco*RI fragment, which was subsequently isolated and cloned, generating pBBP4 (see Figure 2).

Expression of a GST-fusion protein with BSP-binding activity

A clone expressing the N-terminal part of the Bbp protein was constructed by PCR amplification using chromosomal DNA from *S. aureus* strain O24 as a template.

The sense and antisense oligonucleotides used for PCR primers were 5′-CG**GGATCC**GTCTAGGGAACCAAGAAGCT-3′ (corresponding to nucleotides 332–351, as shown in Figure 1), and 5′-ATAGTTTA**GCGGCCGC**AACTTCGCCACCATCTG CATC-3′ (corresponding to nucleotides 2943–2923 in Figure 1), in which the sequences in bold-face indicate *Bam*HI and *Not*I sites inserted into the 5′- and 3′-ends respectively. The underlined nucleotides and the indicated positions in the respective primer correspond to the sequences of the *bbp* gene (see Figure 1). The PCR program was set up as follows: 4 min at 94 °C, followed by 29 cycles of 1 min at 94 °C, 1 min at 60 °C and 4 min at 72 °C. *S. aureus* O24 genomic DNA served as the template in the PCR reaction (Perkin–Elmer thermocycler). The approx. 2.7-kb amplified *Bam*HI/*Not*I fragment was subcloned into a pGEX-5X-2 expression vector (Amersham Pharmacia Biotech) that had been digested with *Bam*HI and *Not*I, and treated with CIP. Competent cells of *E. coli* strain XL-1 Blue (Stratagene) were transformed with the ligation mix, and spread on to a LA plate [Luria–Bertani medium supplemented with 1.5 % (w/v) agar and 50 μg of ampicillin/ml]. The LA plate also contained 20 μg/ml of X-gal and 100 μg/ml of isopropyl β-D-thiogalactoside in order to facilitate the identification of recombinant clones. (In the GST vector, the insert is fused with a part of the plasmid encoding GST, which facilitates purification using glutathione–Sepharose 4B column chromatography.) The fusion protein called GB1 was purified by following the manufacturer's instructions (Amersham Pharmacia Biotech).

Binding assays

Bacteria were grown overnight at 37 °C in TSB, harvested by centrifugation and washed twice with PBS. Bacterial cells (10^{10} cells/ml) were suspended in buffer B3 containing 0.1 % (w/v) ovalbumin. ^{125}I-Labelled BSP [(2–4) × 10^7 c.p.m./μg] was added to the bacterial suspension in a total volume of 0.5 ml. The tubes were coated with 5 % (w/v) BSA in PBS in order to avoid bacterial attachment to the tube walls; the incubation mixture was then rotated 'end-over-end' at 4 °C for 90 min. The reaction was stopped by layering 100 μl of the incubation mixture on to a 10 % Percoll gradient, followed by centrifugation. The supernatant was discarded and the radioactivity associated with the pellet was quantified in a gamma counter and compared with 100 μl of non-centrifuged bacterial solution.

PAGE analysis

The lysates obtained from *E. coli* cells, as well as the purified fusion proteins, were subjected to SDS/PAGE in separation gels consisting of 12 % (w/v) acrylamide, with stacking gels of 3 % (w/v) acrylamide. The samples were dissolved in sample buffer [4 % (w/v) SDS/4 % (w/v) sucrose/0.0625 M Tris/HCl, pH 8.8] and boiled for 3 min before electrophoresis. Protein bands were made visible by silver staining. After separation by SDS/PAGE, material was transferred on to nitrocellulose sheets (membrane filters, BA 83 0.2 μm; Schleicher and Schuell, Dassel, Germany), and incubated in NaCl/P_i supplemented with 2 % (w/v) ovalbumin at 20 °C for 1 h to inhibit non-specific binding. Sheets were incubated with ^{125}I-labelled BSP (10^5 c.p.m./ml overnight at 4 °C under slow agitation), transferred to X-ray film, exposed and subsequently developed.

Dot blot procedure

Proteins to be investigated (10 μl/dot) were immobilized on a Hybond filter previously soaked in buffer B3, and subsequently incubated with ovalbumin to block the filter. Radiolabelled BSP in buffer B3 supplemented with 0.1 % (w/v) ovalbumin was added at a concentration of 50 ng/ml and incubated for 2 h. After washing in Tris-buffered saline (TBS) with 0.2 % (w/v) Tween 20, filters were placed on X-ray film and exposed overnight at −70 °C, before the film was developed.

Identification of antibodies in serum using a microtitre assay

Serum samples were taken from patients suffering from staphylococcal arthritis or osteomyelitis, as well as from patients with septicaemia or endocarditis. Control patients suffered from febrile viral infections, as well as one patient with an abscess harbouring Gram-negative anaerobic bacteria. Microtitre plates were coated with the fusion protein pGB1, teichoic acid and α-toxin, respectively, in a total volume of 50 μl of buffer B3 or PBS, at indicated concentrations, at 4 °C overnight. The wells were blocked by the addition of 100 μl of 5 % (w/v) BSA, washed three times with TBS containing 0.2 % (w/v) Tween 20, and serum was then added in various dilutions in TBS with 0.2 % Tween, followed by an incubation at 37 °C for 2 h. The wells were washed three times, then Protein A–alkaline phosphatase was added, incubated for 2 h at 37 °C and, after washing a further three times, alkaline phosphatase substrate (*p*-nitrophenol; Sigma) was added. The microtitre plates were analysed in an ELISA reader at 405 nm. Positive and negative controls obtained from BioSys Inova were included as controls with all three antigens; values > 1.0 were considered to be positive for the three antigens.

RESULTS

Identification of peptide sequences in Bbp from *S. aureus* strain O24

The seven peptide sequences obtained by in-gel digestion of the 97000-M_r BSP-binding protein purified from LiCl-extracted material from *S. aureus* strain O24 are shown by underlined areas in Figure 1. The order of the peptide sequences in the protein was not known; therefore a set of degenerate oligonucleotides corresponding to the respective peptides were synthesized, and used for PCR in various combinations to amplify a gene fragment encoding a part of the *bbp* gene. This was

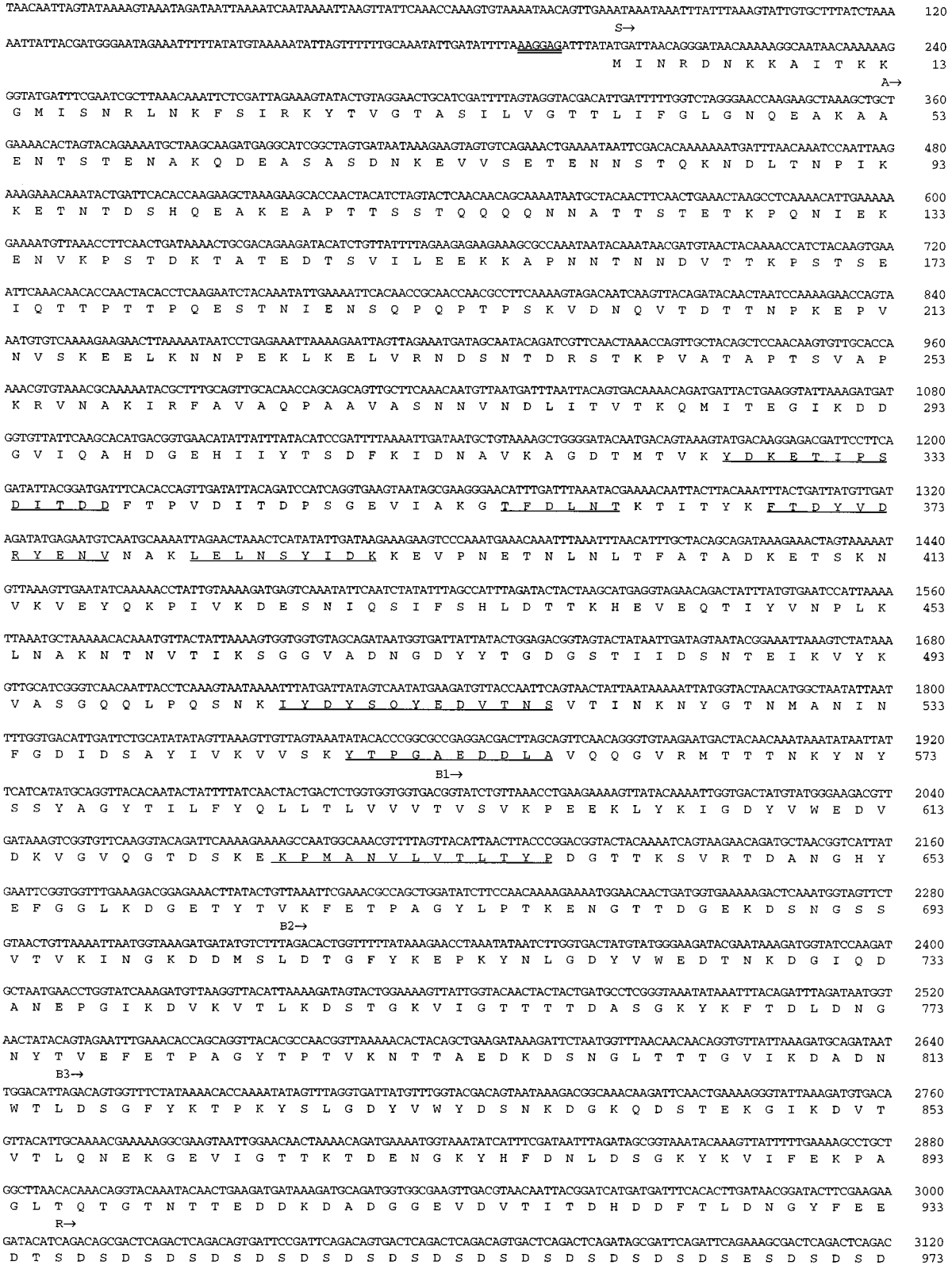

Figure 1 For legend see opposite page

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AGTGACTCAGATTCAGATAGCGATTCAGACTCAGATAGTGATTCAGATTCAGAAAGCGACTCAGATTCAGAAAGCGATTCAGACTCAGATAGCGATTCAGACTCGGATAGCGACTCAGAC   3240
 S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  E  S  D  S  D  S  E  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D   1013

TCAGATAGCGACTCAGATTCAGACTCAGACTCAGACAGTGATTCAGACTCAGATAGCGATTCAGATTCAGACAGTGACTCAGACTCAGACAGTGACTCAGATTCAGATAGCGACGACAGT   3360
 S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D   1053

TCAGATTCAGATAGCGACTCAGACTCAGATAGCGACTCAGACTCAGACAGTGACTCAGATTCAGACAGCGATTCAGACTCAGATAGCGACTCAGATTCAGACAGTGACTCAGACTCAGAT   3480
 S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D   1093
                                                                ←R
AGCGATTCAGATTCAGACAGTGACTCAGACTCAGATAGCGATTCAGATTCAGATGCAGGAAAACATACACCTGTGAAACCGATGAGTGCTACTAAAGACCATCACAATAAAGCAAAAGCA   3600
 S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  S  D  A  G  K  H  T  P  V  K  P  M  S  A  T  K  D  H  H  N  K  A  K  A   1133

TTACCAGAAACAGGTAGTGAAAATAACGGCTCAAATAACGCAACGTTATTTGGCGGATTATTCGCAGCATTAGGTTCATTATTGTTATTTGGTCGTCGTAAAAAACAGAATAAGTAATTT   3720
 L  P  E  T  G  S  E  N  N  G  S  N  N  A  T  L  F  G  G  L  F  A  A  L  G  S  L  L  L  F  G  R  R  K  K  Q  N  K  *      1171

GCTTAACCAAACCAGGTCCGTGAGGCCTGGTTTTTTATTTCAAAATTTATGAAATGTATCTTATGTTTGAGATTAAATAGTGCAATATTTTAAAAGATATAACATACATCAACATAAATT   3840

TAATTCATCTTT                                                                                                              3852
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Figure 1 Complete nucleotide and amino acid sequences of the *bbp* gene from *S. aureus* strain O24

A putative ribosomal-binding site (RBS) is shown by double underlining. Underlined sequences represent peptides determined by amino acid microsequence analysis of purified BSP-binding protein from *S. aureus* O24. The signal sequence (S), the A region (A) and repeat regions (B1, B2 and B3) are shown, as well as the 'R' region, indicating the highly repetitive region consisting of SD residues. The LPXTG motif assumed to be involved in cell-wall anchoring is shown in bold-face type, and the translational stop codon is marked by an asterisk.

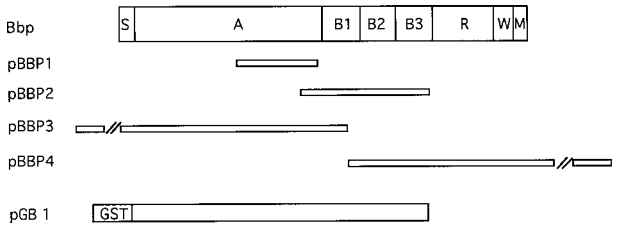

Figure 2 Schematic representation of Bbp of *S. aureus* O24 DNA, and alignment of inserts from both subclones of pBBP1–pBBP4 and a clone encoding a GST-fusion protein, pGB1, of Bbp

The plasmid pBBP1 was the initial PCR product obtained using the primers *obp1* and *obp2*. The pBBP2, pBBP3 and pBBP4 plasmids were produced after colony hybridization using the insert of pBBP1 as a probe. S is the signal sequence; A, the A region; B1, B2, and B3, B-motifs of the B region; R, the repetitive region; W, the cell-wall-spanning region; M, the hydrophobic membrane-spanning region. The pGB1 reveals a GST-fusion protein containing the A-region and the repetitive B-region of Bbp. The primers *obp1* and *obp2* represent the peptide sequences YDKETIPSDITDD and YTPGAEDDLA respectively.

achieved by a combination of oligonucleotide primers *obp1* and *obp2*.

Isolation of the *bbp* gene

Using the degenerate oligonucleotide primers *obp1* and *obp2*, together with chromosomal DNA from strain O24 as the template, it was possible to amplify a DNA fragment of $\approx$ 700 bp. After ligation into a plasmid vector, a clone called pBBP1 harbouring the PCR amplified fragment was isolated (Figure 2). DNA sequence analysis showed that the insert contained one open reading frame. It was found that six out of the seven peptide sequences were present in the deduced amino acid sequence encoded by the isolated fragment (Figure 1). This finding led us to the conclusion that the insert in pBBP1 originates from the gene encoding the BSP-binding protein. Thus, in order to isolate the complete gene, called *bbp*, Southern blot analysis of chromosomal DNA from O24 was performed using the approx. 700-bp fragment as a probe. This approach generated two overlapping fragments that were cloned separately into a plasmid vector generating pBBP2-3 (Figure 2). To obtain the 3′-end of the gene, the insert of pBBP2 was used as a probe to isolate and clone an approx. 4-kb *Eco*RI fragment, generating pBBP4. DNA sequence analysis showed that the *bbp* gene (Figure 1) contained an open reading frame of 3513 nucleotides, starting with an ATG codon at position 201 and ending with a TAA codon at position 3715. The encoded protein thus contains 1171 amino acids, and has a predicted molecular mass of 127 kDa. The deduced amino acid sequence of the Bbp protein contains an N-terminal, typical signal peptide and a possible signal cleavage site between amino acids 51 and 52. Following the signal sequence, there is a region, designated as region A, of 580 amino acids (Figures 1 and 2). This region is followed by an approx. 100-amino-acids-long unit repeated three times, with each unit called B1, B2 and B3 respectively (Figures 1 and 2). Further downstream, a highly repetitive region of 176 amino acid residues composed of tandemly repeated Asp and Ser residues, called R, follows (also Figures 1 and 2). The C-terminal part contains typical features of cell-surface proteins from staphylococci and streptococci, such as putative wall-associated and membrane-spanning regions, as well as the cell-wall-anchoring Leu-Pro-Xaa-Thr-Gly (LPXTG) motif [25,26].

Protein Bbp shows sequence similarities to Sdr proteins of staphylococci

Sequence comparisons reveal that the deduced amino acid sequence of Bbp is similar to that of the SdrE protein of *S. aureus* and protein Fbe, a fibrinogen-binding protein of *S. epidermidis* (Figure 3A). The A region of Bbp shows 76% and 68% identity with the corresponding regions in SdrE and Fbe respectively. The consensus motif TYTFTDYVD found in Bbp is also present in SdrE, Fbe and other members of the Sdr family, except that Thr-367 is replaced by Lys-367 in Bbp of *S. aureus* (Figure 3A). Concerning the A region of Fbe, the N-terminal part of this region is dissimilar to the corresponding parts of the A regions of the respective SdrE and Bbp proteins. A comparison of the A regions of SdrE and Bbp reveals that the dissimilarity is more localized to their C-termini. The B region of Bbp, as for SdrE, is composed of three B motifs, which display a very high degree of

Figure 3 Multiple alignment of deduced amino acid sequence of the A region of *S. aureus* strain O24 Bbp, *S. aureus* Newman SdrE and *S. epidermidis* Fbe (A), and alignment of the repetitive B regions of *S. aureus* O24 Bbp and *S.aureus* Newman SdrE (B)

(**A**) The identical residues are enclosed in boxes and the consensus motif TYTFTDYVD is indicated by the dotted line. The dashes indicate that the sequences are of unequal length, but are matched according to the best alignment of the different sequence stretches. (**B**) The conserved residues are indicated by asterisks, and an EF-hand motif is underlined. Note that the start and the end of each B repeat in the Bbp protein has been adjusted to fit the definition of the B repeats in the SdrE protein [31].

identity of 95–96 % between these two different proteins (Figure 3B), although the three B motifs within the Bbp molecule are less similar to each other (Figure 3B).

BSP binding activity of recombinant Bbp

To study the BSP-binding properties of Bbp, the GST-fusion and -expression system was used. By PCR amplification, the major part of the *bbp* gene encoding the A and repetitive B regions was subcloned into the expression vector pGEX-5X-2, generating the clone called pGB1 (Figure 2). Material from sonicates of *E. coli* was analysed by SDS/PAGE, and, following purification on a glutathione–Sepharose column, a GST-fusion protein of apparent M_r 150000 was detected, interacting with radiolabelled BSP after transfer to nitrocellulose sheets (Figure 4). By using *E. coli* lysates containing the vector only, it was shown that purified GST did not react with radiolabelled BSP (Figure 4). The influence on BSP binding to *S. aureus* O24 cells by addition of potential inhibitors, such as affinity-purified GB1 fusion protein, GST alone and lysates from *E. coli* clones, was investigated in a binding assay. Potential inhibitors were added in dilutions to the incubation mixture before addition of radiolabelled BSP, and the mixture was incubated as described in the Materials and methods section. Inhibitory activity was compared with BSP binding to staphylococcal cells in buffer B3 alone, which then was set as 100 % binding. The fusion protein was shown to inhibit the binding of ^{125}I-labelled BSP to cells of *S. aureus* strain O24 in a concentration-dependent manner, whereas lysate of *E. coli* harbouring only the GST vector or purified GST alone did not (Figure 5). The interaction between ^{125}I-labelled BSP and the purified GB1 fusion protein was investigated further by using a

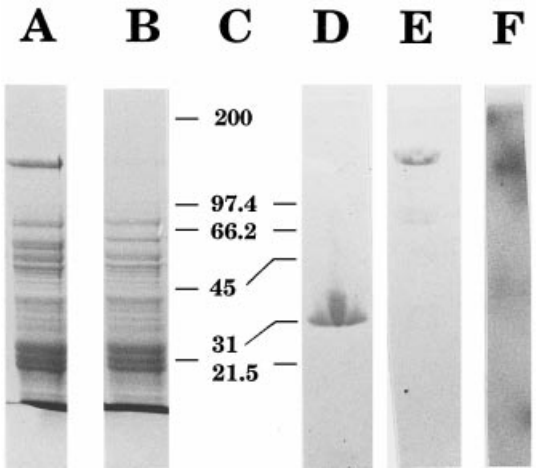

Figure 4 SDS/PAGE and Western blot analyses of the GST-fusion protein of Bbp

Material for analysis was released by sonication of *E. coli*. Lysate from *E. coli* containing the GST-fusion Bbp is shown before (lane A) and after (lane B) passage through a glutathione–Sepharose column. Lane E shows material eluted from this column by glutathione; lane C indicates Bio-Rad molecular weight markers (in kDa); lane D shows material eluted from the glutathione–Sepharose column after passage of lysate from *E. coli* containing only the GST vector without Bbp. Lane F shows material from lane E transferred on to nitrocellulose, and incubated with ^{125}I-labelled BSP.

direct binding assay in a dot blot experiment. Lysates from *E. coli* clones, as well as purified GST alone, were studied in parallel with the GB1 fusion protein; 10 μl of each protein solution was

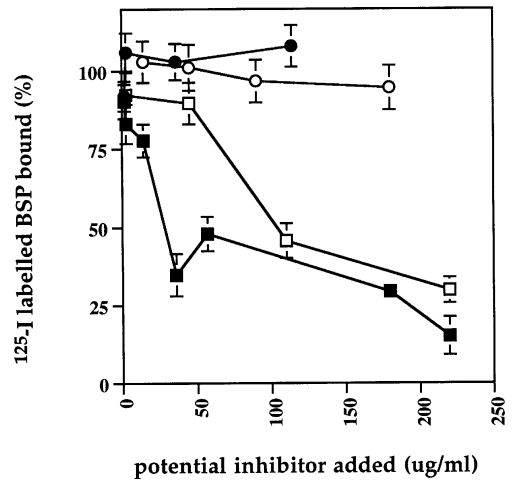

Figure 5 Inhibition of binding of radiolabelled BSP to *S. aureus* O24 cells by addition of potential inhibitors

Potential inhibitors investigated were as follows: ■, affinity-purified GB1 lysate from *E. coli* containing the GST-fusion Bbp; □, GB1; ●, GST alone; ○, *E. coli* containing empty GST vector.

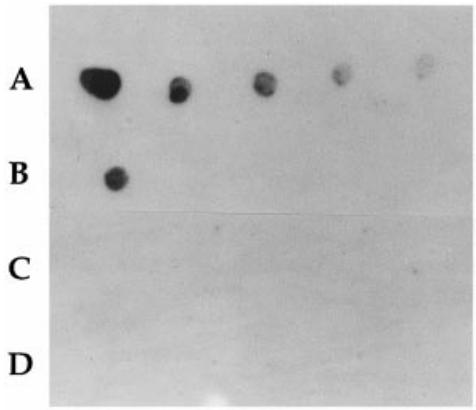

Figure 6 Incubation with ^{125}I-labelled BSP of material applied on nitrocellulose

Lanes were loaded as follows: A, affinity-purified fusion protein of Bbp (GB1); B, lysate from *E. coli* containing the GST-fusion protein of Bbp; C, material eluted by glutathione from glutathione–Sepharose after passage of lysate of *E. coli* containing the GST vector only; D, lysate from *E. coli* containing the GST vector only. In each dot, 10 μl was applied; 1:5 dilutions were performed in lane A.

immobilized on a filter and incubated with ^{125}I-labelled BSP in a dot blot system. Binding of radiolabelled BSP to immobilized proteins was detected by exposure on X-ray film (Figure 6). A concentration-dependent interaction was identified between GB1 and BSP. Lysate of *E. coli* harbouring the pGB1 also bound ^{125}I-BSP, as shown for one dilution only (Figure 6), because of the multitude of proteins included in the lysate, and therefore concentration measurements of the fusion protein were not determined. Furthermore, the binding signal was found to decrease as the lysate was diluted (results not shown). No interaction was found between BSP and GST alone or lysate of *E. coli* harbouring the GST vector without the *bbp* gene (Figure 6).

Table 1 Antibody titres against staphylococcal antigens in human serum

Microtitre assays were performed using the Bbp fusion protein GB1, teichoic acid (TA) and α-toxin as antigens. Sera were diluted 50-fold and the absorbance at 405 nm was measured at different time intervals. The cut-off level for positive values was taken as $A_{405} > 1.0$. Values shown are means for duplicate samples.

	Coating antigen		
Patient no./diagnosis	GB1	TA	α-Toxin
1/Septic arthritis, *S. aureus*	2.23	2.44	2.23
2/Endocarditis, osteitis, *S. aureus*	2.19	2.20	1.87
3/Osteitis, *S. aureus*	1.91	1.81	2.28
4/Endocarditis, *S. aureus*	0.40	2.02	1.24
5/Endocarditis, *S. aureus*	0.18	2.52	0.22
6/Septicaemia, *S. aureus*	0.25	1.29	1.41
7/Abscess, *Bacteroides*	0.51	1.84	0.56
8/Viral meningitis	0.16	1.67	0.95
9/Viral infection	0.22	0.97	0.24

GB1 fusion protein used as antigen in an ELISA

The fusion protein GB1 was used as antigen in a serological microtitre assay to determine if this protein is immunogenic and, if so, whether a difference in antibody response could be detected in patients suffering from severe staphylococcal infection, as opposed from patients suffering from other non-staphylococcal infections. Teichoic acid and α-toxin, the commonly used antigens in staphylococcal serology, were used for comparison. The cut-off level for a positive signal was set at a measurement of A_{405} of > 1.0. Three patients suffering from staphylococcal bone and joint infection generated antibodies against the GB1. In contrast, patients suffering from other severe staphylococcal diseases, such as endocarditis and septicaemia, did not (Table 1). Two patients suffering from acute viral infections, as well as one patient suffering from an abscess from Gram-negative bacteria, did not raise antibodies against the fusion protein. Antibodies raised against teichoic acid were found in sera from all three of the latter patients (Table 1). Although anti-(teichoic acid) antibody titres were higher in patients with staphylococcal infection, this antigen did not discriminate between infection with bone and joint localization and endocarditis/septicaemia (Table 1). Anti-(α-toxin) antibodies were present in sera from patients with staphylococcal disease, but no significant difference in titre was noted between the different localizations of staphylococcal infection (Table 1).

DISCUSSION

In the present study, we have reported on the cloning and sequencing of a novel staphylococcal gene, named *bbp*, that encodes a protein with affinity for BSP. The deduced protein consists of 1171 amino acid residues. We have previously characterized a BSP-binding protein, of M_r 97000 from *S. aureus* strain O24 [36]. All peptide sequences obtained from the 97000-M_r protein were recovered in the sequence encoded for by the *bbp* gene. A GST-fusion protein containing a major part of the Bbp bound ^{125}I-labelled BSP; the fusion protein also inhibited binding of this ligand to staphylococcal cells. The molecular mass of the cell bound protein encoded by the *bbp* gene (118 kDa) is higher than that of the BSP-binding protein purified from *S. aureus* cells, which is 97 kDa. A protein with a molecular mass of 127 kDa might migrate as an 97000-M_r peptide in SDS/PAGE; alternatively, the 97000-M_r protein might be a proteolytic

fragment of the native protein. Nevertheless, taking all data together, it is possible to conclude that the *bbp* gene encodes the previously characterized 97000-M_r BSP-binding protein of *S. aureus* strain O24.

The Bbp displays similarities in structure and organization to other cell-surface-associated proteins from *S. aureus* that interact with ECM components. These include FnBPA and FnBPB [11], ClfA and ClfB [12,13,27,29], and Cna [10,12,13,27]. In all of these proteins, an N-terminal signal sequence (S) is followed by a region consisting of both unique and repetitive sequences that confer the ECM-binding activity [17]. The cell-wall-spanning region (W) is followed by an LPXTG motif, a sequence shown to be involved in cell-wall anchoring [25,26]. A similarly organized cell-wall-associated protein, which is a fibrinogen-binding protein, was recently characterized from *S. epidermidis* [32]. The latter protein contains an SD-repeat sequence, which is also present in the protein reported here. The Bbp protein contained an SD-repeat sequence of 176 residues; this protein can therefore be placed among the Sdr protein family [31]. Some of these proteins have been reported to bind to specific plasma components such as fibrinogen (ClfA and ClfB), whereas the binding properties of others remain unclear [31]. A member of this protein family reported recently, SdrD, possesses calcium-binding activity [39], but no other function of this protein has been reported. Binding of BSP to staphylococcal cells was shown to be higher in the presence of Ca^{2+} [36]. It may therefore be possible that the conformation of the BSP-binding protein is influenced by the presence of Ca^{2+}, yielding a higher affinity for BSP when Ca^{2+} is present. The Bbp was most similar to SdrE from *S. aureus* strain Newman [31], showing 76% identity in the A region. No ligand-binding activity has yet been reported for SdrE. The SD-repeat sequence seemed not to be essential for BSP-binding activity on the basis of the fact that a fusion protein containing the A and B regions, but not the SD-repeat sequence, inhibited binding of ^{125}I-labelled BSP to staphylococcal cells, and bound ^{125}I-BSP after immobilization of the fusion protein on nitrocellulose. The ligand-binding domain of other matrix-binding proteins from staphylococcal cells has been shown to be located either in the A region, such as in the case of Cna [40] and ClfA [12], or in C-terminal repetitive domains (the D regions) of FnBPA [11,41]. The exact position of the BSP-binding domain of Bbp has yet to be identified, although, by using fluorimetry, the addition of recombinant BSP or staphylococcal binding BSP peptides (at concentrations of up to four equivalents of peptide) [35] did not influence the fluorescence of 8-anilinonaphthalene-1-sulphonate (ANS)- or pyrene-labelled B1–B5 repeats from SdrD (Cecilia Rydén, unpublished work; personal communications from Elisabet Josefsson, University of Gothenburg, Sweden, and Jos Cox, University of Geneva, Switzerland), indicating that the B repeats are not involved in the interaction between BSP and staphylococcal cells. As reported previously, BSP binding to staphylococcal cells was not influenced by addition of plasma or fibrinogen even at high concentrations [36], indicating that the BSP-binding protein, although similar to ClfA and ClfB, has a different ligand specificity. Binding of ^{125}I-BSP to staphylococcal cells is inhibited by neither fibronectin nor IgG [7], whereas the 97000-M_r protein inhibited this binding, albeit not completely [36].

Sera from patients suffering from bone and joint infections contained antibodies against Bbp fusion protein, in contrast with sera from patients suffering from septicaemia or endocarditis. Although investigated only in a limited number of patients, these results point to the fact that Bbp is immunogenic. Furthermore, the results are compatible with the hypothesis that Bbp contributes to the pathogenesis of staphylococcal osteomyelitis and arthritis. In a previous study, we demonstrated that BSP is selectively bound by *S. aureus* cells isolated from patients with osteomyelitis or septic arthritis, but not by isolates from patients diagnosed with endocarditis [6]. The possibility of using Bbp as an antigen for early screening of suspected staphylococcal osteomyelitis/septic arthritis to determine the nature of a potential infectious site in bone tissue or joints is limited, since it is often difficult to obtain cultures from these sites. The physical examination of the patient often does not reveal the localization of a bone infection, and there are no reliable diagnostic serology tests available yet for these diseases. Further studies are needed to assess the clinical usefulness of Bbp-based serology.

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