Sorting a *Staphylococcus aureus* phage display library against *ex vivo* biomaterial

Joakim Bjerketorp, Anna Rosander, Martin Nilsson, Karin Jacobsson and Lars Frykberg

Department of Microbiology, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden

A phage display library made from *Staphylococcus aureus* DNA was sorted against a central venous catheter (CVC) that had been removed from a patient 2 days after insertion. After the first panning, approximately 50 % of the clones encoded proteins known to interact with mammalian proteins. After the second and third panings, fibrinogen-binding and β₂-glycoprotein I (β₂-GPI)-binding phage particles were clearly dominating. Proteins adsorbed to different CVCs were investigated using specific antibodies. Among the proteins probed for, fibrinogen was most abundant, but, interestingly, β₂-GPI was also detected on all tested CVCs.

**INTRODUCTION**

Biomaterial is widely used in modern medicine, both as a permanent replacement, e.g. prosthetic heart valves, and for short time usage, e.g. intravenous catheters. In contact with blood, a number of different plasma proteins adhere to the implanted material. Protein adsorption can cause problems such as activation of the coagulation system, complement activation and promotion of bacterial colonization (Courtney et al., 1994). The adsorption depends on the material itself, but is also a dynamic process, thus the composition of adsorbed proteins may change over time (Vroman et al., 1980). Skin-associated *Staphylococcus epidermidis* and *Staphylococcus aureus* are among the bacteria most frequently isolated from biomaterial infections (von Eiff et al., 2002).

*Staphylococcus aureus* encodes a large number of proteins that interact with host proteins, so-called receptins (Kronvall & Jönsson, 1999). Many are well characterized, but additional receptins have been predicted from the genome sequence (Kuroda et al., 2001). Several plasma proteins promote *S. aureus* adherence to polymeric surfaces, i.e. fibrinogen (Fg), fibronectin (Fn), laminin, vitronectin, thrombospondin and von Willebrand factor (vWF) (Herrmann et al., 1988, 1991, 1997; Vaudaux et al., 1984; Fuquay et al., 1986). Studies of *S. aureus* adherence to biomaterial coated with various plasma proteins have given valuable information on the host proteins with which *S. aureus* interacts. However, information on which receptin(s) mediates the adherence is limited since *S. aureus* encodes several receptins with similar binding specificity. In addition, such studies depend on the bacterial growth conditions and growth phase and may not totally reflect the *in vivo* situation.

Since its introduction by Smith in 1985 (Smith, 1985), phage display has been a very useful method for identifying protein–protein interactions. A foreign protein is displayed at the phage surface as a fusion to one of the phage coat proteins. Usually the minor (pIII) or major (pVIII) coat protein is used for this purpose. To minimize disturbance of phage functions, systems that allow fusion to only some of the coat proteins, e.g. phagemid vectors, are frequently used. Insertion of foreign DNA into gene-VIII-based phagemid vectors results in multivalent display, but the number of displayed polypeptides will vary considerably for different polypeptides (Malik et al., 1996, and references therein). Phage libraries made from bacterial DNA consist of phage particles that together theoretically display all proteins encoded by the bacterial genome. Simultaneously, the genetic information for the displayed polypeptide is contained in the phagemid vector inside the phage particle. Such libraries can be used for identification of receptins by panning against various ligands (Jacobsson & Frykberg, 1995, 1996, 1998). Here, we have sorted an *S. aureus* library against a central venous catheter (CVC) removed from a patient 2 days after insertion.

**METHODS**

**Biomaterials.** Discarded polyurethane CVCs (Becton Dickinson) were obtained from Uppsala University Hospital, Sweden. The CVCs had been removed from five different patients who had had a CVC inserted in a major blood vessel for periods varying between 2 and 18 days. The CVCs were frozen, and before use, gently washed in PBST (PBS + 0.05 % Tween 20) to remove any blood remnants.
Phage library. The library used is described by Bjerketorp et al. (2002). It was constructed from genomic DNA of S. aureus strain Newman, consisted of $1 \times 10^6$ clones and had a titre of $1.5 \times 10^8$ c.f.u. ml$^{-1}$. The library was made in the gene-VIII-based phagemid vector pG8SAET (Jacobsson & Frykberg, 2001), which should result in multivalant display of the foreign polypeptides on the phage surface. The vector contains a screening tag (E-tag) in reading frame with gene VIII but out-of-frame with the signal peptide. The cloning site is located just in front of the E-tag and hence the expression can be restored if an inserted DNA fragment, with an open reading frame, corrects the frameshift. Expression of the E-tag is used to follow enrichment for putative correct clones.

Escherichia coli strain TG1 was grown in Luria–Bertani (LB) broth or on LA plates (LB broth supplemented with 1.5 % agar). Ampicillin (50 mmol l$^{-1}$) was added when appropriate (LA-Amp). All bacterial incubations were done at 37 °C.

Pannings. A 400 µl portion of the phagemid library was mixed with casein (Sigma) at a final concentration of 100 µg ml$^{-1}$ and incubated with a ~5-mm-long piece of CVC removed from a patient 2 days after insertion. After panning at room temperature (RT) with shaking for 4 h, the CVC was washed thoroughly with PBS and bound phages were eluted with 400 µl elution buffer (0.05 M sodium citrate, pH 2.9). The eluate was neutralized with 50 µl 2 M Tris-buffer, pH 8.7, and 0.1–50 µl was added to 25 µl stationary phase E. coli TG1, together with LB broth to a final volume of 200 µl. The infection proceeded for 20–30 min at RT before the suspension was spread on LA-Amp plates. The resulting colonies were screened (see below) and infected with helper phage R408 (Promega) for production of enriched phage stocks for repanning, as described by Jacobsson et al. (2003). Repannings were carried out as described above, but for only 2 h.

Panning against in vitro coated microwells (Maxisorp; Nunc) was done as described by Jacobsson & Frykberg (1998). Wells were coated for 1 h with human plasma (Uppsala University Hospital, Sweden) diluted 20 times in coating buffer (50 mM NaHCO$_3$, pH 9).

Individual phage stocks were prepared by R408 infection of bacterial cultures of clones Nbm21, Nbm29 and Nbm69 (Table 1). The titre of each phagemid stock was adjusted to 10$^8$ c.f.u. ml$^{-1}$. Pannings with these phage stocks against ~5 mm pieces of CVCs (either unused, or removed from patients after 6, 9, 13 or 18 days) or against pure ligands in coated wells (Maxisorp; Nunc) was done as described by Jacobsson & Frykberg (1998). Wells were coated for 1 h with human plasma (Uppsala University Hospital, Sweden) diluted 20 times in coating buffer (50 mM NaHCO$_3$, pH 9).

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Screening and sequencing of phagemid clones. After each round of panning, colonies were transferred to nitrocellulose membranes (Schleicher & Schuell) and subsequently screened for expression of the E-tag with anti-E-tag antibodies (Amersham Biosciences) as described by Jacobsson et al. (2003). Phagemid DNA from E-tag-positive clones was prepared with the Qiagen Miniprep kit according to the manufacturer’s instructions. The inserted DNA fragments were sequenced using the DYEnamic ET Terminator Cycle Sequencing Premix kit (Amersham Biosciences) and the samples were analysed using the ABI 377 DNA Sequencer (Perkin Elmer) according to the manufacturer’s instructions. The Vector NTI computer software (Informax) was used for handling of the sequences obtained.

Analysis of proteins adsorbed to the ex vivo biomaterials. Pieces of CVCs (~5 mm) were boiled in 40 µl sample buffer (62.5 mM Tris/HCl, pH 6.8; 10 % glycerol; 2 % SDS; 5 % β-mercaptoethanol; and 0.01 % bromophenol blue) for 5 min and the released proteins were subjected to SDS-PAGE using the PhastSystem with 4–15 % gels (Amersham Biosciences). For analysis of β$_2$-GPI, pieces of the same CVCs were boiled in a non-reducing buffer (composition as above, but without the β-mercaptoethanol), since this protein contains 11 disulfide bridges (Kato & Enjoji, 1991), and the monoclonal antibodies only reacted weakly with the reduced form (unpublished observation). Proteins were diffusion blotted (PhastSystem at 65 °C for 40 min) onto nitrocellulose membranes (ECL; Amersham Biosciences), which were treated with the Quantix Western Blot Signal Enhancer kit (Pierce). Thereafter the membranes were blocked for 30 min in PBST containing casein at a concentration of 1 mg ml$^{-1}$. Proteins were detected using horseradish peroxidase (HRP)-labelled antibodies against Fn (DakoCytomation), Fg (DakoCytomation) or IgG (ICN). β$_2$-GPI was detected using an anti-β$_2$-GPI monoclonal antibody (Chemicon) followed by HRP-labelled anti-mouse antibodies (Amersham Biosciences). Bound HRP-labelled antibodies were visualized using BM Blue POD substrate (Roche).

RESULTS AND DISCUSSION

Sorting the phage library against ex vivo biomaterial

The S. aureus phage display library was panned in three consecutive cycles against a CVC that had been removed from a patient 2 days after implantation. The phage display approach does not discriminate between cell-wall-located and secreted receptins, and is independent of the expression level of the receptin. Thus in theory all bacterial proteins interacting with the biomaterial may be found. After each panning, colonies were counted and screened for E-tag expression. In the first panning, 9 × 10$^3$ phagemid particles ml$^{-1}$ were recovered, and in the second and third pannings the number of phagemid particles was 4 × 10$^3$ and 6 × 10$^3$ ml$^{-1}$, respectively. The frequency of E-tag-positive clones increased from 12 to 38 and to 78 % in the consecutive pannings, showing that an enrichment for clones with open reading frames occurred.

From each panning, approximately 50 E-tag-positive clones were isolated and the DNA sequences of the inserts were determined. Clones were considered to represent an authentic interaction if overlapping inserts of a gene were found in two or more clones, or if a clone encoded a known receptin. After the first panning, approximately 50 % of the clones were correct. In the second and third pannings, only one background clone was found. However, despite the large number of clones investigated, no novel receptin was identified.

Receptins identified

The receptins identified were: coagulase (Coa), an Fg- and prothrombin (PT)-binding protein with the ability to coagulate plasma; protein A, an IgG- and vWF-binding protein; Efb, a Fg-binding protein; FnbpA/B, two similar Fn- and Fg-binding proteins; and Sbi, an IgG- and β$_2$-GPI-binding protein (Table 1). The binding domains in these proteins have been mapped, and therefore it is possible to
deduce which binding property is displayed by a selected phage particle. However, it is possible that additional, so far unidentified, binding domains may exist or that a domain might bind more than one protein. The majority of isolated phage encoded either Fg-binding or /C2262-GPI-binding poly-peptides. Part of the coa gene was found in 35 clones, all encoding the Fg-binding repeat region, and only in one clone was the PT-binding domain also present. Altogether, 74 clones contained parts of the sbi gene. Interestingly, 51 of these clones encoded only the part known to bind /C2262-GPI, while the others encoded polypeptides with the ability to bind both IgG and /C2262-GPI.

Protein Sbi contains a signal peptide and has been found associated with the surface of S. aureus. However, the protein does not contain the typical LPXTG motif and the trans-membrane domain found in most cell-wall-bound proteins, suggesting a different type of association to the surface. Still, similar to cell-wall-spanning domains, Sbi contains a repetitive domain with proline residues repeated every fifth amino acid (Zhang et al., 1998, 1999).

### Table 1. Different clones found after sorting an S. aureus genomic phage display library against an ex vivo CVC

<table>
<thead>
<tr>
<th>Clone</th>
<th>Encoded receptor*</th>
<th>Amino acids</th>
<th>Panning cycle†</th>
<th>Deduced binding ability</th>
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<tr>
<td></td>
<td></td>
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<td>Total no. of clones</td>
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<td></td>
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<td>50</td>
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</table>

*References: 1, Signäs et al. (1989), Jönsson et al. (1991), Wann et al. (2000); 2, Uhlen et al. (1984), Hartleib et al. (2000); 3, Bodén & Flock (1994); 4, Kaïda et al. (1987), Kawabata et al. (1986); 5, Zhang et al. (1998, 1999).
†The numbers indicate how many times a specific clone was isolated after each panning cycle.

Panning against microwells coated in vitro with plasma

The results from the panning experiments against ex vivo biomaterials suggest that β2-GPI is easily accessible for Sbi displayed on the phage surface. One possible explanation for this could be that β2-GPI is deposited later than Fg and Fn. As a comparison, plasma proteins were coated onto microwells for 1 h, and the phage display library was panned in two cycles against the wells. Following the first and second pannings, 24 E-tag-positive clones were analysed. After the first panning, nine clones contained the coa gene, five the fnbB gene, three the spa gene (encoding protein A) and one the efb gene, while six clones were considered as background.

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After the second panning, only clones encoding Fg-binding proteins were found. These results suggest that Fg is the main protein recognized by *S. aureus* proteins after a short *in vitro* coating.

**Proteins released from *ex vivo* biomaterials**

The high number of clones with proteins recognized by proteins were found. These results suggest that Fg is the main coating.

To our knowledge it has not previously been shown that β₂-GPI is deposited on biomaterial. β₂-GPI is a single-chain 50 kDa protein, and although present in relatively high amounts in serum (~0.2 mg ml⁻¹), the biological function of β₂-GPI is not completely clear. It binds to negatively charged substances such as heparin and phospholipids, and is structurally related to regulators of the complement system (Kato & Enjyoji, 1991; Steinkasserer et al., 1991). β₂-GPI has been reported to have several biological functions, such as promoting clearance of liposomes and maybe foreign particles from the circulation, but also effects on the coagulation system have been reported (Chonn et al., 1995; Nimpf et al., 1986). The mechanism for the anticoagulating activity of β₂-GPI was suggested in a recent publication, which showed that β₂-GPI binds to factor XI and inhibits its activation (Shi et al., 2004).

**Panning with phage stocks against *ex vivo* biomaterials and pure proteins**

Considering bacterial adhesion to biomaterial, the total amount of protein deposited is less important than its accessibility on the surface. To confirm the accessibility of Fg, IgG and β₂-GPI on different CVCs, phage stocks were made from three relevant clones. After adjusting their titres, the binding to CVCs as well as to pure proteins immobilized in microwells was investigated. Although CVCs were derived from different individuals and the insertion time varied, this did not seem to greatly affect the ability of the phage clones to bind to the CVCs. As expected from the Western blot data, the Fg-binding clone Nbm69 demonstrated good binding to all CVCs, but ~100 times more phage particles bound to pure Fg (Fig. 2a). The binding capacity of Nbm29, encoding part of protein A, varied to some extent between CVCs, but it was much higher than to an uncoated CVC (Fig. 2b). The phage particles also bound to pure IgG, but no binding to rvWF was observed (Fig. 2b). The lack of binding to rvWF may be caused by differences between rvWF and vWF purified from serum; alternatively, the conditions in our experiments might not be ideal to detect this interaction. The *sbi*-derived Nbм21 phage bound well to β₂-GPI but, as expected, not to pure IgG (Fig. 2c). Interestingly, these phage particles also bound well to all tested CVCs (Fig. 2c), showing that β₂-GPI is accessible on all tested CVC surfaces, further strengthening our findings regarding β₂-GPI in the Western blot (Fig. 1e). A negative control phage stock containing the vector pG8SAET showed no binding to either of the CVCs (data not shown).

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**Fig. 1.** Analysis of proteins released from *ex vivo* biomaterial. Coomassie brilliant blue stained SDS-PAGE gels run under reducing (a) and non-reducing conditions (d), and Western blots of the corresponding gels incubated with antibodies against human Fg (b), IgG (c) and β₂-GPI (e). Lanes: 1, proteins from a CVC removed after 6 days; 2, after 9 days; 3, after 13 days; 4, after 18 days. M, Molecular mass standard (Bio-Rad broad range: 200, 116, 97, 66, 45, 31, 22, 14 and 7 kDa).
Even though the signal strengths in the Western blots of proteins released from the CVCs may not show the exact relation between protein amounts, the signals imply that Fg is present in higher amounts than \( \beta_2 \)-GPI (Fig. 1). However, the Fg-binding and \( \beta_2 \)-GPI-binding phage particles bound to the CVCs in comparable numbers (Fig. 2), thus demonstrating that it is the surface accessibility rather than the total amount of protein that is of importance for the phage binding. The binding of phage particles to pure Fg was approximately 25 times higher than to pure \( \beta_2 \)-GPI, and since similar numbers of Nbm69 and Nbm21 phage particles bound to the CVCs, this suggests that \( \beta_2 \)-GPI is actually more accessible than Fg on the CVCs.

**Concluding remarks**

When Dickinson *et al.* (1995) investigated the adherence of *S. aureus* to *in vitro* coated glass surfaces under flow, it was concluded that adherence to Fg or plasma almost exclusively depended on the Fg-binding protein clumping factor (CF). Francois *et al.* (2000) also demonstrated the importance of CF for *S. aureus* binding to Fg-coated polyvinyl chloride (PVC) tubing. However, it was also demonstrated that Fg deposited on the PVC tubing *in vitro* was much more efficient in promoting *S. aureus* adherence than the Fg deposited during haemodialysis. In addition, an *S. aureus* mutant lacking the CF bound in 10-fold higher numbers to the tubing coated during haemodialysis than to control tubing coated with albumin. Similarly, our result from panning against a well coated with plasma *in vitro* yielded mainly Fg-binding phage. A possible explanation for these differences is the over layering of initially adhered Fg by other plasma proteins on the haemodialysis tubing. When the tubing coated during dialysis was subjected to analysis of the protein content, it was suggested that an unidentified protein(s) different from Fg, vitronectin, thrombospondin and vWf was responsible for the additional binding of *S. aureus* to the tubing (Francois *et al.*, 2000). Our panning against the CVCs removed from different patients shows that \( \beta_2 \)-GPI is an easily accessible component, and may be the unidentified target on the haemodialysis tubing above. Thus the ability to bind \( \beta_2 \)-GPI could be one mechanism by which *S. aureus* adheres to biomaterial.

\( \beta_2 \)-GPI has attracted a lot of attention in recent years due to its involvement in autoimmune disease. In antiphospholipid syndrome (APS), autoantibodies are often not directed against phospholipids alone, but against phospholipids bound to \( \beta_2 \)-GPI or to \( \beta_2 \)-GPI only (McNeil *et al.*, 1990; Galli *et al.*, 1990). Although the exact specificity of antiphospholipids (aPL) antibodies is under debate, it has been suggested that the concept ‘aPL antibodies’ needs to be revaluated since the major target is presumably not phospholipids but \( \beta_2 \)-GPI (Li & Krilis, 2003). The cause for the appearance of these autoantibodies is not known, but aPL antibodies are frequently found in conjunction with various infections (McNeil *et al.*, 1991). It has been suggested that a change in conformation of \( \beta_2 \)-GPI upon contact with negatively charged phospholipids or surfaces may be responsible for exposure of autoepitopes (Borchman *et al.*, 1995; Chamley *et al.*, 1999; Wang *et al.*, 2000). In this context, the binding of \( \beta_2 \)-GPI to biomaterial is interesting. It is known that binding of plasma proteins to polymer surfaces can result in a conformational change of the protein (Barbucci & Magnani, 1994). Perhaps as a consequence, haemodialysis patients were found to have a significantly higher incidence of aPL antibodies compared to control groups (Fabrizi *et al.*, 1999; McIntyre & Wagenknecht, 2003). The finding of \( \beta_2 \)-GPI on the surface of CVCs is intriguing, but whether adherence of \( \beta_2 \)-GPI to biomaterials is a general phenomenon needs to be investigated.

Sorting an *S. aureus* phage display library against *ex vivo* biomaterials revealed several possible interactions. However, this type of experiment identifies proteins that bind to the target regardless of the normal localization and function of the protein. Thus it may be possible to find interactions without biological meaning. Still, all recepins identified in this study contain a signal peptide and are extracellular proteins, thus they could be of biological significance. Two of them, Efb and Coa, are secreted, although a small fraction of

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**Fig. 2.** Panning of phage stocks against CVCs removed from patients after a different number of days (white bars) and against microwells coated *in vitro* with pure human proteins as indicated (black bars). (a) Clone Nbm69 displaying part of the Fg-binding protein Coa; (b) Nbm29 displaying part of protein A reported to bind IgG and vWf; (c) Nbm21 displaying the \( \beta_2 \)-GPI-binding region of protein Sbi. None of the phage stocks bound to casein (data not shown).
Coa seems to be surface associated (Bodén & Flock, 1989; McDevitt et al., 1992). Their primary function probably is not adherence; still the binding of these proteins to biomaterial might serve other biological functions. After the initial adherence of *S. aureus* to biomaterial, it is conceivable that the Fg binding of Coa could cause a localized coagulation, which could promote biofilm formation on the biomaterial. Interestingly, EfB and Coa were also found when an *S. aureus* library was sorted against platelets, suggesting that these proteins bind platelets via Fg as a bridging molecule and possibly could aid *S. aureus* adherence to platelets (Heilmann et al., 2002).

The importance of CF for *S. aureus* adherence to Fg has been demonstrated in several publications (e.g. Dickinson et al., 1995; Francois et al., 2000), but CF was not found in this study. Most likely, phages that display CF are out-competed by other binding phages, perhaps due to its large ligand-binding domain. Although the major selection should be for binding to the immobilized ligands, other factors may also influence which phage dominate after panning. For example, it is possible that different phagemid clones replicate to different numbers, or that different polypeptides, with the same binding specificity, might be displayed with different efficiency. Ten different clones encoding parts of the *coa* gene were found after the first and second pannings. Among these, Nbm42 contains almost five complete Fg-binding repeats, but this clone is not found after the second or third pannings. Instead, Nbm69, encoding only two Fg-binding repeats, dominates after the third panning. Clearly factors other than Fg-binding alone are responsible for the dominance of this clone.

Although no new recepin was identified in this study, it was shown that β2-GPI is deposited on the CVCs. The results also showed that with this kind of phage library, only one panning cycle is needed to obtain a high frequency of correct clones. Further pannings resulted in an even higher frequency of correct clones, but much fewer unique clones. Thus in order to find new interactions, it might be better to make only one panning and investigate a larger number of clones.

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