Gene VIII-Based, Phage-Display Vectors for Selection Against Complex Mixtures of Ligands

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ABSTRACT

Selection of shotgun phage-display libraries against complex mixtures of components, such as cells or sera, may result in a high number of nonspecifically binding phage. Consequently, correct interactions may be difficult to identify. To enable discrimination between faithful and nonspecific interactions, a set of eight different gene VIII-based, phage-display vectors were constructed. All vectors contain a "universal" screening tag positioned in such a way that it is only expressed when the inserted DNA encodes an open reading frame, which corrects a shift of reading frames in the vector. A Staphylococcus aureus shotgun phage-display library was made in a stoichiometric mixture of all vectors. After affinity-selection against IgG, one vector completely outcompeted the others. This vector contains the promoter and signal sequence from the gene encoding staphylococcal protein A and one suppressible stop codon immediately upstream of gene VIII. An increase in the frequency of clones expressing the affinity tag in all pannings correlated with selection for ligand-binding clones. This enables detection of putatively correct clones after selection of a shotgun phage-display library both against purified ligands and more complex materials like calf serum.

INTRODUCTION

Since its introduction in 1985 (17), phage display has developed into a very useful technique for studying protein-protein interactions. The amino acids involved in an interaction between two molecules can be identified by using random peptide libraries (RPL) (1–3,5,16). It is also possible to isolate short peptides that mimic interactions occurring in nature, such as peptides that bind to concanavalin (Con) A or streptavidin (3,12,15).

We have previously shown that shotgun phage display can be used to clone ligand binding domains in prokaryotic receptors by affinity-selection against the ligand (7,8). As a model bacterium, we chose *Staphylococcus aureus*, a pathogen responsible for a wide variety of diseases in humans and animals, such as endocarditis, osteomyelitis, wound sepsis and mastitis (4). This bacterium produces several extracellular virulence factors such as alpha-, beta-, gamma- and delta-toxins, toxic shock syndrome toxin (TSST), enterotoxins, leucocidin, proteases, coagulase and clumping factor (6). *S. aureus* also expresses several different surface receptors (adhesins), which interact with host matrix proteins such as fibronectin, vitronectin, collagen, laminin, elastin and bone sialoprotein (13). In addition, staphylococci can bind several serum proteins, e.g., IgG, fibronectin, fibrinogen and trombospondin (13). Some of the genes encoding these bacterial proteins are not yet cloned, and most likely, more interactions between the host and bacteria are to be

identified. The likely importance of such proteins in host/bacterial interactions prompted us to improve methods for their identification.

A shotgun phage display library, made by insertion of randomly fragmented DNA from *S. aureus* into a gene III-based phagemid vector was affinity-selected against IgG and fibronectin (Fn), resulting in 5%–40% positive clones (7). In a gene VIII-based phagemid system (multi-valent display of the fusion protein), this number increased to 75%–100% (8). However, panning against more complex ligands, like cells or immune sera resulted in a background of nonspecifically binding phage (K. Jacobsson and L. Frykberg, unpublished observations). During the development of these cloning systems, an unusual feature was observed: both the gene III and the gene VIII phage-display systems exhibit a shift in reading frame between the inserted DNA and the coding regions within the vector. In the gene III system, the shift occurs at the transition between the 3' end of the insert and the vector; whereas, in the gene VIII system, the shift-site is located at the transition between the signal-sequence in the vector and the 5' end of the insert (7,8). Accordingly, in both cases, ribosomal slippage is required for expression of the fusion protein. Most likely, this apparent selection for inserts in the "wrong" reading frame is a natural way for the system to optimize the production of viable phage by down-regulating the expression of the coat-fusion protein. Unfortunately, in the gene-VIII system, this results in an expression

level of the encoded insert too low for detection in a screening assay with a peroxidase-labeled ligand. This can create problems, since yielding low frequencies of positive clones may require a screening system to identify binding clones. In addition, panning against more complex materials consisting of a mixture of different ligands, like cells and serum, will result in an enrichment of a high number of clones reacting with different, unknown ligands. Thus, no ligand-specific screening system can be used. Therefore, we have developed a set of vectors with a "universal" screening tag out-of-frame with the signal sequence, but in-frame with gene VIII. This design ensures that clones with inserts containing an open reading frame (ORF) that corrects the frame-shift can be detected with labeled human serum albumin (HSA). This means that an increasing fraction of clones expressing the tag will correlate to a selection for the displayed polypeptides. Such an approach can only be used when DNA fragments of random, variable length are inserted into a phage-display vector. In addition, a number of precautions have been taken to avoid selection for clones with inserts out of reading frame, which would hamper detection of the tag.

First, sequences suspected to promote ribosomal slippage in pHEN1 and pG8H6 (7,8) were omitted. Second, 0–3 suppressible stop codons were introduced between the tag and gene VIII to obtain varying expression levels of the full-length fusion protein, but ensuring a high expression of insert and tag (Figure 1). Third, a second set of vectors identical to the ones described above, except for a substituted promoter and signal sequence [from the gene encoding staphylococcal protein A (*spa*)], was constructed (pG8SPA0-3; Figure 1).

A shotgun phage-display library was made in a stoichiometric mixture of all eight vectors, and panning against IgG was used to determine which vector was able to outcompete the others. This library was also affinity-selected against newborn calf serum (NCS). Our data showed that positive clones with affinity for serum components could be identified using labeled HSA, i.e., expression of the screening tag.

MATERIALS AND METHODS

Bacterial Strains and Helper Phage

E. coli MC1061 was used for con-

struction of the vectors, and *E. coli* strain TG1 was used for construction of libraries and production of phage stocks. Phage R408 (Promega, Madison, WI, USA) was used as helper phage. *E. coli* transformants were grown in LB medium supplied with 1% glucose and 50 µg/mL ampicillin.

Construction of the Vectors

All DNA manipulations were performed using standard methods (14), except for ligations for which Ready-To-Go™ T4 DNA Ligase (Pharmacia Biotech, Uppsala, Sweden) was used. Modification and restriction enzymes were from Amersham International (Little Chalfont, Bucks, England, UK) or MBI Fermentas AB (Vilnius, Lithuania). All oligonucleotides were synthesized by Pharmacia Biotech.

The pG8SPA- and pG8LP-phagemid vectors were constructed from pG8H6 (8). Primers Gen83 and G8Bam were used to amplify gene VIII from pG8H6 and simultaneously introducing a *Bam*HI site in front of the gene. The fragment encoding the HSA-binding domain was amplified from pSZG40 (10) using the primer NcoAlb, containing the *Sma*I and *Sna*BI sites for blunt-end cloning, and either AlbStO,

Figure 1. Schematic drawing of (A) the pG8PL and (B) the pG8SPA vectors. The signal sequence and the HSA-binding domain are not in the same reading frame, and thus no HSA-binding polypeptide is produced unless an insert has corrected the shift in reading frame. Cloning sites present in all vectors: *Nco*I, *Sna*BI and *Sma*I/*Xma*I.

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Table 1. Deoxyoligoribonucleotides Used in the Construction of the pG8LP- and PG8SPA-Series of Phagemid Vectors and/or Used in Hybridizations and Nucleotide Sequencing of the Inserts

NcoAlb	Constr/hyb	5'-gat <u>ccc atg gca gta cgt acc cgg ggc</u> gga cat tac agg agc agc ctt gt-3'
AlbSt0	Constr/hyb	5'-gat <u>cgg atc cga cct</u> cag aac tcg gta gag c-3'
AlbSt1	Constr/hyb	5'-gat <u>cgg atc cct</u> aga cct cag aac tcg gta gag ctg a-3'
AlbSt2	Constr	5'-gat <u>cgg atc cct</u> act aga cct cag aac tcg gta gag ctg ata-3'
AlbSt3	Constr	5'-gat <u>cgg atc cct</u> act act aga cct cag aac tcg gta gag ctg ata aaa-3'
Pranc	Constr	5'-cga <u>tcc cat ggt</u> cat cgt gtt gcg cag cat tt-3'
SapPr	Constr	5'-gat cag <u>cgg aag agc</u> cgg ttt taa gcc ttt tac t-3'
G8Bam	Constr	5'-ata <u>ggg atc cga ggg</u> tga cga tcc cgc aaa-3'
Gen83	Constr	5'-ttt ccc <u>gaa ttc</u> atc ggt tta tca gct tgc ttt cg-3'
Salead	Hyb/sequen	5'-tat ctg gtg gcg taa cac ctg ct-3'
Pel	Hyb/sequen	5'-cct att gcc tac ggc agc cgc tgg-3'
H2Stop	Hyb	5'-tga ggt cta gta ggg at-3'
H3Stop	Hyb	5'-agg tct tag tag tag gga-3'
Albp	Sequen	5'-gcc ata ctg ctt tag ttc att gat -3'

Sites for the restriction enzymes used in the cloning procedure are underlined.

Table 2. Number of Eluted Phagemid Particles (cfu) and Fraction of IgG- and HSA-Binding Clones after Panning Against Human IgG.

	Eluate (cfu) ^a	HSA Bind ^b	IgG Bind ^b
1st panning	1 × 10 ⁵ (1 × 10 ⁵)	20 (39)	29 (44)
2nd panning	3 × 10 ⁷ (5 × 10 ⁷)	58 (67)	74 (82)
3rd panning	nd (2 × 10 ⁸)	nd (62)	nd (92)

^aNumber of colony-forming units. Figures in parenthesis represent the second experiment.
^bPercentage ligand-binding clones of 150 screened.
 nd = not done.

AlbSt1, AlbSt2 or AlbSt3 to introduce 0, 1, 2 or 3 amber stop codons, respectively. A fragment containing the promoter region and the signal sequence from the *spa* gene was amplified from the plasmid pSPA15 (18) using primers Pranc and Sap1pr. The fragments obtained were cut with *Bam*HI/*Eco*RI, *Nco*I/*Bam*HI and *Sap*I/*Nco*I, respectively. First, the pG8LP-series was constructed by digesting pG8H6 with *Eco*RI/*Nco*I, followed by treatment with calf intestine alkaline phosphatase (CIAP). Subsequently, the fragments encoding gene VIII and the HSA-binding domain were ligated into the vector, and the ligation mixture transformed into *E. coli* MC1061. Plasmids prepared from ampicillin-resistant colonies were analyzed by restriction digestion. Plasmids yielding the expected

restriction pattern were further characterized by nucleic acid sequence determination. Next, the vector pG8LP0-3 was digested with *Sap*I and *Nco*I followed by CIAP-treatment. The *spa* fragment was ligated into the respective vector, and the ligation mixtures were transformed into *E. coli*. Plasmids were prepared from ampicillin-resistant colonies and analyzed as described above. A polymerase chain reaction (PCR)-induced point mutation in the protein A signal sequence, resulting in an amino acid exchange from Lys to Arg in the third position, was found in all four pG8SPA vectors.

Construction of the Library

The library was constructed as described previously (7,8). In short, a

mixture of 10 µg of each of the eight vectors was digested with *Sna*BI and dephosphorylated. Eight micrograms of the vector mixture were ligated with 8 µg of *S. aureus* fragments (300–2000 bp) in 13 tubes of Ready-To-Go Ligase (Pharmacia Biotech). The ligated material was phenol- and chloroform-extracted, ethanol-precipitated and dissolved in 20 µL of H₂O. Twenty 1-µL aliquots of the ligation mixture were electrotransformed into *E. coli* TG1, and the transformed cells were grown overnight in 200 mL LB medium supplied with 1% glucose and 50 µg/mL ampicillin. A 4-mL aliquot of the overnight culture was infected with helper phage (multiplicity of infection [MOI] 20), mixed with 100 mL 0.5% soft agar and poured onto 20 LA plates (LB medium with 1.5% agar and 50 µg/mL ampicillin). After overnight incubation, the phages were eluted from the soft agar.

Panning Procedures

Pannings were carried out as described (7,8) with some minor modifications. Microwell[®] plates (Maxi-Sorp[™]; Nalge Nunc International, Copenhagen, Denmark) were coated with human IgG (Kabivitrum, Stockholm, Sweden) at a final concentration of 100 µg/mL in 0.05 M sodium car-

bonate, pH 9.7, and blocked with PBS-T (phosphate-buffered saline with 0.05% Tween® 20). NCS (Life Technologies, Inchinnan, Scotland, UK) was diluted 20 times in coating buffer. The library and the stocks used for repannings were pre-incubated with 0.1 mg/mL HSA (Sigma Chemical, St. Louis, MO, USA) for 15 min before addition to the wells to inhibit nonspecific binding to potentially contaminating HSA. In some pannings against NCS, the library/stock was pre-incubated with human IgG (100 µg/mL) to avoid enrichment for protein A. After panning for 3 h at room temperature, the wells were rinsed 30 times with PBS-T, bound phage were eluted with 200 µL 50 mM Na-citrate/140 mM NaCl, pH 2.0, followed by neutralization with 25 µL 2 M Tris-HCl, pH 8.7. The eluate was used to infect *E. coli* TG1 and plated for determining the number of phagemids and for amplification as described previously (8). To determine how the phagemids replicate in the absence of affinity-selection, a 10-µL portion of the library was re-amplified twice.

After each panning against IgG, 150 colonies were picked to 9 plates (LB medium with 1.5% agar and 50 µg/mL ampicillin) in identical patterns. All eight vectors without inserts were included as controls. After pannings against calf serum, the colonies were picked to 3 or 4 plates in identical patterns.

Screening

Colonies from all pannings were screened for binding of horseradish peroxidase (HRP)-labeled HSA (5 µg/mL) or IgG (5 µg/mL) as described (7). After pannings against NCS, some filters were screened for fibronectin-binding clones using human fibronectin (10 µg/mL) and rabbit anti-human fibronectin antibodies, followed by detection with HRP-labeled goat anti-rabbit antibodies (all from Sigma Chemical) according to the manufacturer's instructions.

Hybridizations

Colonies were transferred to nitrocellulose filters and processed accord-

ing to standard procedures (14). Hybridizations were carried out at temperatures 5°–10°C below melting temperature (T_m), and washings were done in 0.2% sodium dodecyl sulfate (SDS)/2× standard saline citrate (SSC) or 0.5% SDS/6× SSC (20× SSC = 3.0 M NaCl and 0.3 M Na-citrate) at 5°C below T_m . The specificity was confirmed by exposure of wet filters to a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA) and, if background signals remained, the washing was continued at temperatures increased in steps of 1°C.

Nucleotide Sequencing of the Displayed Inserts

Plasmid DNA was prepared using Wizard™ Minipreps (Promega), and the sequence of the inserts was determined as described previously (8), using primers Albp and Salead or Pel (Table 1). The PC-gene program (IntelliGenetics, Mountain View, CA, USA) was used for handling of the sequences obtained.

RESULTS AND DISCUSSION

Construction of the Vectors

We reported earlier that shotgun phage-display cloning of bacterial adhesins results in 75%–100% correct clones after two pannings in a gene VIII-based system (8). However, this result was obtained after panning against a pure ligand, and the binding by the receptor was mediated by a short domain. Panning against more complex materials, like cells or immune serum, resulted in a high background of non-specifically binding phage (K. Jacobsen and L. Frykberg, unpublished results). In addition, the shifts in reading frame found in almost all clones after affinity-selection hamper the identification of positive clones with a labeled ligand. Therefore, we decided to construct new improved gene VIII-based phage display vectors, which should be able to express the inserted DNA in correct reading frame. Hence, the sequences encoding the *c-myc* tag and the histidine tail, which we believe promote ribosomal slippage in pHEN1 and

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pG8H6, respectively, were omitted. In addition, an increasing number of suppressible amber stop codons (0, 1, 2 or 3) were placed in front of gene VIII, which is expected to give a reduced expression of the fusion protein. This reduced expression should circumvent the selection for clones with inserts that are out-of-reading-frame. Furthermore, a screening tag was introduced in such a way that it enables detection of putative positive clones irrespective of the ligand used in the panning. The HSA-binding tag was introduced out-of-reading-frame with the signal sequence, but in-frame with gene VIII, and with a cloning site positioned immediately upstream of the tag (vectors pG8PL0-3; Figure 1A). Thus, an appropriately inserted DNA fragment with an ORF will restore the ORF and, accordingly, the tag will be expressed. The amber stop codons were placed downstream of the screening tag so that the expression of the insert and tag seg-

Table 3. Fraction of Clones Assigned to the Different Vectors after Pannings Against IgG or After Repeated Amplifications of the Library in the Absence of Any Selection

	pG8SPA Vectors				pG8LP Vectors			
	0 ^a	1 ^a	2 ^a	3 ^a	0 ^a	1 ^a	2 ^a	3 ^a
IgG 1st	19 (15)	29 (44)	24 (28)	6 (7)	4 (0)	7 (5)	5 (0)	6 (2)
2nd	19 (18)	56 (65)	16 (17)	2 (0)	1 (0)	3 (1)	1 (0)	1 (0)
3rd	17 (17)	74 (78)	5 (5)	1 (1)	0 (0)	1 (0)	0 (0)	2 (0)
Uns.1st	76				24			
2nd	22	22	23	20	4	3	1	5
3rd	28	26	17	25	1	1	1	1

^aNumber of stop codons in the vector.

Figures show the percentage of clones contained in the respective vector of all 150 colonies screened. Figures in parenthesis show the percentage of clones contained in the respective vector of the IgG-binding clones among the 150 colonies screened.

ment should remain high. Thereby, it should be possible to detect expression of the insert and/or the tag. Earlier pannings of *S. aureus* shotgun phage-dis-

play libraries against IgG resulted in a significant fraction of clones expressed from the *spa* promoter, in which the protein A signal sequence promoted

Table 4. Number of Eluted Phagemid Particles after Panning Against NCS and the Frequency of Bacterial Colonies Expressing Affinity for IgG, HSA or Fibronectin

Panning	Wash (cfu) ^a	Eluate (cfu) ^a	HSA Bind ^b	IgG Bind ^b	Fn Bind ^b
1st	6 × 10 ⁴ (1 × 10 ⁴)	4 × 10 ⁴ (3 × 10 ⁴)	3 (5)	0.7 (1)	nd (7)
2nd	1 × 10 ⁵ (3 × 10 ⁴)	3 × 10 ⁵ (3 × 10 ⁵)	18 (47)	5 (23)	nd (17)
3rd	2 × 10 ⁵ (2 × 10 ⁵)	1 × 10 ⁷ (7 × 10 ⁷)	44 (59)	89 (67)	nd (0)
3rd inh ^c			51 (nd)	0.7 (nd)	19 (nd)

^aNumber of colony-forming units per mL. Two separate experiments are shown, the second of which is shown in parenthesis.
^bPercentage ligand-binding clones of 150 screened.
^cThird panning repeated in the presence of IgG.
nd = not done.

transport of the coat protein to the membrane (Reference 8 and unpublished observation [K. Jacobsson and L. Frykberg]). This result suggested that the *spa* promoter and signal sequence might be suitable in a phage-display vector. Therefore, a set of four vectors was constructed, where the *lac* promoter and *peIB* leader in pG8PL0-3 were exchanged for the *spa* promoter and signal sequence (Figure 1B).

Construction of a Library in a Mixture of Eight Vectors

All eight vectors, mixed in stoichiometric amounts, were used for construction of a *S. aureus* shotgun phage-display library. The final library consisted of 3 × 10⁸ independent clones, 88% of which contained an insert, and had a titre of 3 × 10¹⁰ colony-forming units (cfu)/mL. After ligation, the frequency of clones that had obtained HSA-binding fortuitously, i.e., carried an inserted DNA fragment with an ORF and with a length that corrected the shift in reading frames in the vector, was approximately 3% (data not shown). In the propagated library, after infection with helper phage, the frequency of HSA-binding clones had decreased to approximately 1%. Thus, an increase to more than 1% HSA-binding clones after panning should indicate a selection for the displayed polypeptide.

After Panning Against IgG, Clones Expressing the Activated Tag Correlate with Positive Clones

To confirm the correlation between selection for ligand-binding clones and an increasing fraction of HSA-binding

clones, the library was panned against human IgG in two separate experiments. After each panning, 150 colonies were characterized with respect to binding of HRP-labeled IgG and HSA (Table 2). After three consecutive pannings, the number of bound phagemid particles had increased 1000-fold. After the third panning, 92% of the bacterial colonies obtained from the eluted phagemids could be detected with labeled IgG, and out of these, 2/3 also bound HSA (Table 2). This indicates that 92% of the inserts are fused in-frame with the signal sequence, but a significant fraction (1/3) still showed a shift in reading frames between the inserted DNA and the HSA-binding domain. The nucleotide sequence of five IgG-positive/HSA-negative clones was determined. As expected, they showed a shift in reading frame between the insert and the tag (data not shown). Nevertheless, the reading frame shifts were found in only 33% of the clones, as determined by the ability of the bacterial colonies to bind IgG and HSA, compared with 48 out of 48 clones obtained with the previous vector (pG8H6) (8). It is possible that panning against another ligand may result in a different frequency of inserts out-of-frame depending on how the encoded polypeptide affects the *E. coli* cell. Labeled HSA was not uniformly bound to different colonies, which was not unexpected since different lengths of the inserted DNA might result in different expression levels and/or could be due to decreased accessibility of the HSA-binding domain due to the remainder of the expressed polypeptide. Also, it cannot be excluded that some of the weak HSA signals could be caused by a

frame shift, which is efficiently corrected by ribosomal slippage.

Competition Between the Vectors

The panning experiments described above were also used for selection of the vector with superior performance. For this purpose, the signal sequence and the number of stop codons were determined in the 150 colonies characterized after each panning (Table 3). Already after one round of selection, an enrichment for the pG8SPA vectors was seen. After additional consecutive pannings, which resulted in an increasing number of IgG-binding clones, the pG8SA1 vector (*spa* promoter and signal sequence with one stop codon) outcompeted the others. After three pannings, 78% of the IgG-binding clones were identified as pG8SPA1 derivatives. No IgG-binding clones were found in any of the vectors with the *lac* promoter and *peIB* leader (Table 3). This result was unexpected, since the shift in reading frame found earlier must reduce the expression level of fusion protein and, hence a repressible promoter (e.g., *lac* promoter) was expected to be preferred. The preference for the pG8SPA vectors could conceivably depend on the signal sequence, which might be more efficient for translocation of the fusion protein to the membrane.

To determine if there was any difference in how well the phagemid vectors replicated, they were subjected to three rounds of amplification without affinity-selection. Surprisingly, the pG8SPA vectors also now outcompeted the pG8PL vectors. After three amplifications, 96% of the clones were identified

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as pG8SPA vectors, and only small differences in the number of stop codons were found (Table 3). This result suggests that the pG8SPA vectors are either more efficient in replication or in packaging of the phagemid.

Panning Against Newborn Calf Serum

The library was also panned against NCS to test whether a selection as reported above also hold true for interactions with a complex mixture of different ligands and whether the "universal screening tag" can be used to identify "correct" clones after such pannings. Note that the tag was reported not to bind bovine serum albumin (BSA) in a dot blot assay (10). However, in this multi-valent display system, the tag does react with BSA, although with an affinity much lower than for HSA. Thus, the binding to BSA can conveniently be inhibited by incubating the

library with HSA before panning (data not shown). In all pannings, phage stocks were pre-incubated with HSA at a final concentration of 100 µg/mL for 15 min before addition to the coated wells. After three consecutive pannings against NCS in two separate experiments, the number of binding phage had increased approximately 1000-fold (Table 4). After each panning, 150 colonies were screened for HSA-, IgG- and, in some cases, for fibronectin (Fn)-binding. The frequency of HSA binding clones increased from approximately 4% after the first panning to about 50% after the third panning (Table 4). After the first panning, the fraction of IgG-binding clones was very low, but after three pannings, it ranged from 65%–89%. In the second experiment, the number of Fn-binding clones was also determined. After the first panning, more Fn-binding than IgG-binding clones were found, but in the second and third pannings, the Fn-binding clones were outcompeted, mainly, by IgG-binding clones.

In the first experiment, almost exclusively, IgG-binding clones were recovered after three consecutive pannings. To avoid this selection, the stock used in the third panning was pre-incubated with human IgG, which reduced the fraction of IgG-binding clones from 89% to less than 1%, while the fraction of HSA-binding clones was unaffected. This indicates that pre-incubation of the phage stock with free ligand efficiently blocks binding of phage to the same serum component. Thereby, an enrichment for other clones is obtained. These results clearly demonstrate that the enrichment of serum protein-binding clones can be followed as an increase in HSA-binding clones, as shown in Table 4. In the first experiment (second panning), 15 HSA-binding clones that did not bind IgG were characterized by DNA sequencing. Fourteen of these contained DNA encoding Fn-binding proteins (9,11). The 15th clone contained an insert derived from *sbi* gene, the second IgG-binding protein in *S. aureus* (7,19). Interestingly, the fragment isolated here lacks the IgG-binding domain (data not shown). The same *sbi* fragment was found in 15 clones, judged as negative for Fn binding but positive for HSA binding after

the third panning with IgG inhibition. In the second experiment, 16 clones, likewise negative for IgG and Fn binding, were sequenced. Fourteen clones contained overlapping inserts derived from the *sbi* gene, only one of which contained the IgG-binding domain (data not shown). This strongly suggests that the SBI protein binds a second serum component beside IgG.

CONCLUSIONS

After three consecutive pannings against IgG, with the library made in a mixture of all eight vectors, the pG8SPA1 vector, i.e., *spa* promoter and signal sequence combined with one stop codon, had outcompeted the others. Since this result was obtained after panning against one ligand only, it cannot be excluded that other ligands would enrich for a different vector, depending on whether a lower or higher number of fusion proteins per phage particle is beneficial. Still, we suggest that the pG8SPA1 vector should be used for future construction of shotgun phage-display libraries.

Furthermore, correct clones could be identified by detection of the screening tag after affinity-selection against a complex mixture of molecules. The use of a universal affinity tag expressed only in clones containing an ORF that corrects the frame shift introduced in the vector, is extremely useful for identification of putatively correct clones. It significantly reduces the number of clones to investigate even if the background of nonspecifically binding phage to the immobilized ligands is high. Maybe more importantly, we show here that this can be done already after the first panning. This is desired, since consecutive pannings may sometimes result in enrichment for a single type of clone, as illustrated by the sequencing of 15 clones containing the same part of the *sbi* gene after three pannings against NCS. Similarly, three pannings against NCS, without inhibition with free IgG, resulted in enrichment for almost exclusively IgG-binding clones. Hence, less avid interactions may remain undetected if successive enrichment steps are used.

Infection of the host by pathogenic

bacteria involves multiple interactions. Both extracellular and surface-located bacterial proteins interact with soluble as well as cell and matrix-associated proteins of the host. The identification of such bacterial proteins are important for the understanding of the infection process. Panning against serum resulted not only in enrichment for IgG- and Fn-binding polypeptides but also identified an unexpected interaction between the SBI protein and a so far unidentified serum component.

Libraries constructed from bacterial DNA in the vectors described here, can be used for selection against the host structures with which bacteria interact, such as serum components, cells, tissues or implants removed from patients, and binding clones can be identified using the tag. The vectors and the techniques described in this paper can be directly applied to all prokaryotes and, with some modifications, also to eukaryotes.

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