An Improved Phage Display Procedure for Identification of Lipopolysaccharide-Binding Peptides

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We successfully implemented several modifications to the regular phage display procedure and significantly improved the lipopolysaccharides-binding properties of the peptides selected. Specifically, the number of biopannings was increased and peptides with consensus sequences were obtained. A dual selection procedure (referred to as subtractive panning) was used to simultaneously select for the desired target and deselect for an undesired target, thereby increasing the binding specificity. In addition, binding and washing conditions in the subtractive panning were also modified to favor the selection of peptides with higher binding strength. As a result, two peptides, ASFPPAF and SSHTISF, were identified with much improved binding properties compared to those selected with regular panning. The binding specificities of these two peptides, as measured by the ratio of phages bound to the desired and undesired targets, were severalfold higher than previously reported. These modifications could easily be implemented with many other target molecules, indicating the general applicability of the procedure.

Introduction

Phage display technology allows the presentation of large peptide and protein libraries on the surface of filamentous phage and permits selection of peptides and proteins with high affinity and specificity (*1*). Using this technology, peptides have been identified for species-specific recognition of *Bacillus anthracis* spores (*2*) and peptides that bind specifically to the H7 flagellin of *E. coli* (*3*).

Lipopolysaccharides (LPSs) are the outermost structure of Gram-negative bacteria (*4*). LPS consists of three components (Figure 1) (*4*): Lipid A, a core oligosaccharide, and a highly variable region called O antigen, typically a repeating structure of an oligosaccharides chain. It is this structure that gives each organism a distinct signature. In *E. coli*, over 175 serotypes (classified on their O-antigens) were identified (*5*). LPS plays a critical role in pathogenesis and mediates the septic shock that kill millions every year. Peptides that bind to LPSs could potentially neutralize their effects. They could also be used as a less expensive, more stable alternative to antibodies in a wide variety of bioassays with applications in food, agriculture, biotechnology, and medical diagnostics (*6*). Therefore, there is great interest in identifying peptides that bind LPSs. Several studies have demonstrated that some short peptides possess some binding specificities to LPSs. (*7*−*9*).

In this study, we demonstrate that by incorporating simple modifications in the phage display procedure, peptides having consensus sequences and significantly improved binding properties could be identified.

Materials and Methods

Materials. Lipopolysaccharides of *Escherichia coli* O111:B4, *E. coli* O55:B5, *Salmonella typhimurium*, BSA (bovine serum albumin), IPTG (isopropyl-β-D-thiogalactopyranoside), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), TMB (3,3′,5,5′-tetramethylbenzidine), and PEG (poly(ethylene glycol))-8000 were purchased from Sigma (St. Louis, MO). Ph.D.-7 Phage Display Peptide library with a complexity of 2.8×10^9 was purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase/anti-M13 monoclonal conjugate was procured from Amersham Pharmacia Biotech (Piscataway, NJ).

Regular Panning. The LPSs were coated on a Nunc Polysorp 96-well microtiter plate (Nunc, Denmark) by incubating them overnight at a concentration of 100 μg/mL in a carbonate coating buffer (Na_2CO_3 0.15%, $MaCl_2 \cdot 6H_2O$ 0.1%, $NaHCO_3$ 0.3%, pH 9.6) at 4 °C. After removing LPS, wells were blocked with a 1% BSA blocking buffer at 4 °C for 1 h, upon which they were washed with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) six times. The phage library (2×10^{11} pfu/well) was first incubated in a well not coated with LPS for 1 h at room temperature, upon which the unbound fraction was transferred to LPS-coated wells. After 1 h of incubation, the wells were washed with TBST 10 times with 0.1% Tween for the first panning, and 0.5% Tween for subsequent pannings. The bound phages were then eluted with 100 μL of glycine-HCl (0.2 M, pH 2.2) for 10 min and then neutralized with 15 μL of Tris-HCl (1 M, pH 9.0). The eluate was then amplified by infection to *E. coli* ER2738. The phages were purified by precipitation with PEG-8000/NaCl at 4 °C as described by the manufacturer's instructions and titrated using LB/IPTG/Xgal agar plates. The titrated phage was then diluted to 2×10^{11} pfu/well in TBST and applied as the input phage for subsequent pannings.

Subtractive Panning. To screen for a superior binder with minimal reaction to undesired LPSs, a subtractive strategy was used in the subsequent phage display experiment. The panning procedure was the same as the regular panning described above, except that an additional incubation step with LPS-O111 was added in each panning, and the unbound fraction was used for panning with LPS-O55. In addition, other modifications were introduced to increase binding affinity. A comparison of the two procedures is shown in Figure 2.

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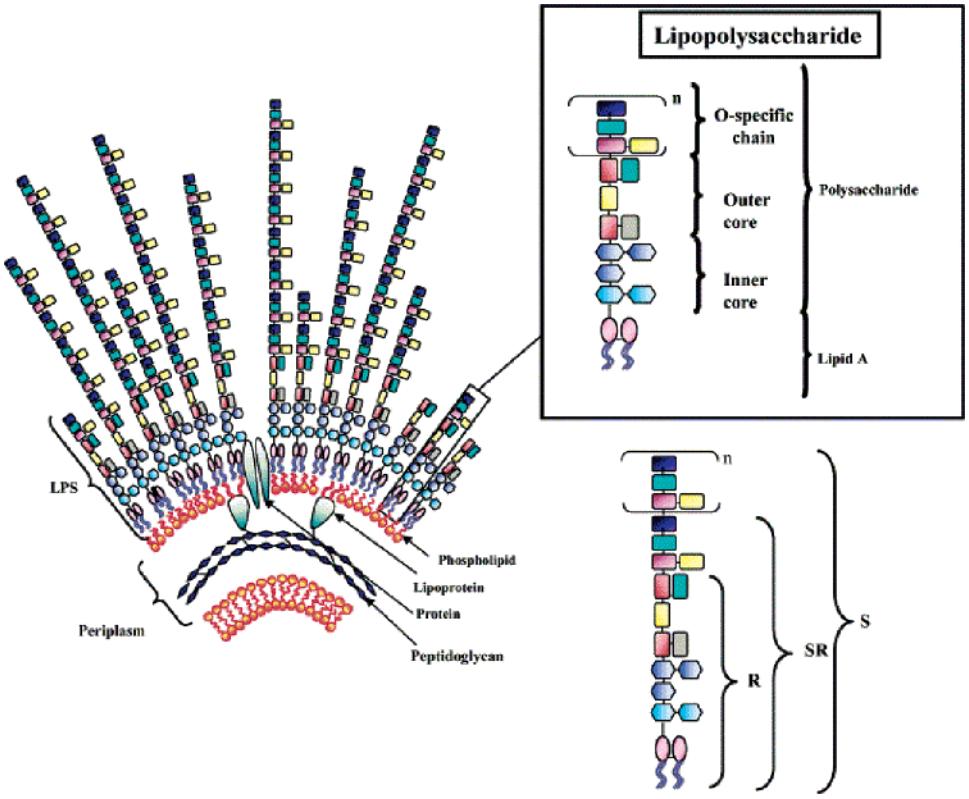
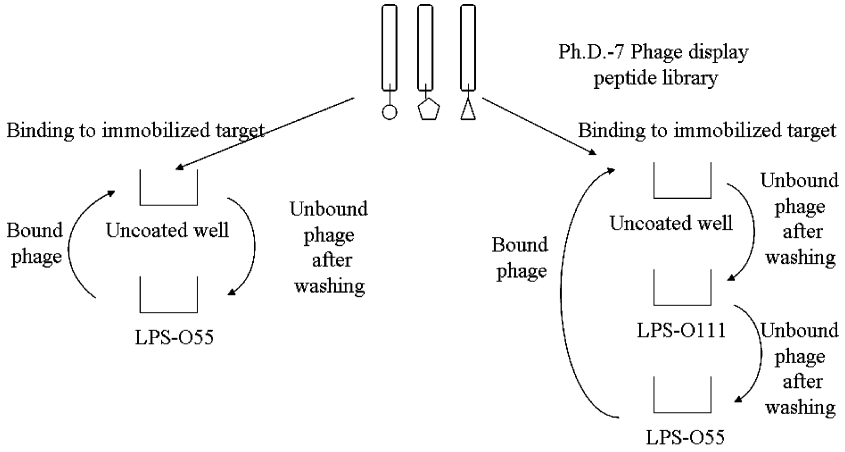


Figure 1. Illustration of Gram-negative bacteria lipopolysaccharide structure (adopted from Caroff et al., 2003 with permission from Elsevier).



Panning condition					
Round	1	2	3	4	5
Phage binding (min)	60	60	60	60	60
TBST (%)	0.1	0.5	0.5	0.5	0.5

Regular panning

Panning condition					
Round	1	2	3	4	5
Phage binding (min)	60	60	60	40	40
TBST (%)	0.1	0.5	0.5	0.5	1.0

Subtractive panning

Figure 2. Schematic representation of the two phage display procedures: regular vs subtractive pannings.

DNA Sequence Analysis. The unamplified eluate from the last round of biopanning was plated on LB/IPTG/Xgal plates. Ten well-isolated plaques were randomly picked and amplified. The single-stranded DNA from isolated phage clones was extracted with Qiagen QIAprep Spin M13 kit (Valencia, CA) and sequenced with 96 gIII primer (5′-CCCTCATAGTTAGCGTAACG-3′). The nucleotide sequence of selected clones was determined at the DNA Sequencing Core Lab of the Georgia Institute of Technology. Sequences were analyzed using DNAMAN software (Lynnon Corp., Quebec, Canada).

Characterization of Binding Clones by Phage ELISA. The selected phages were amplified, purified by PEG/NaCl precipitation, titrated, and subsequently subjected to phage ELISA. The LPSs were coated as described above, then washed with PBS three times, and blocked with 1%BSA in PBS. Appropriately diluted phage (10^9-10^{10} pfu/100μL) was incubated in both coated and uncoated wells for 2 h at room temperature. The wells were then washed with PBST five times and incubated with HRP/Anti-M13 antibody conjugate (1:5000 in blocking buffer) for 1 h at room temperature. After washing three times with PBST and twice with water, wells were reacted with 100 μL TMB substrate solution at room temperature for 20 min. Reaction was stopped by adding 1 M sulfuric acid, after which absorbance was measured at

Table 1. Summary of Peptide Sequences Identified by Biopanning against *E. coli* O55:B5 LPS

biopanning	sequences	no. of clones with identical sequence
regular panning	NIMRNTW	4 out of 10
	AVPRASF	4 out of 10
	LGSYNNA	2 out of 10
subtractive panning	ASFPPAF	4 out of 6
	SSHTISF	2 out of 6

450 nm using a Victor III microplate reader (Perkin-Elmer, Boston, MA).

Results and Discussion

Increasing the Number of Rounds of Biopanning To Obtain Peptides with Consensus Sequences. Screenings of LPS-binding peptides using a phage display library have been reported (*10*−*13*). On only one occasion, using epoxy beads as carriers for LPS, were homologous sequences achieved against the LPS of *Salmonella enteritidis* (*10*). Other studies with LPS immobilized on microtiter plates failed to enrich phage clones with consensus sequences. These biopanning experiments were carried out with a commercial kit (Ph.D.-12 Phage Display Peptide Library, New England Biolab). Typically three biopannings were carried out using the Phage Display Peptide Library (from the kit) with the target LPS molecule of *E. coli* O55:B5 immobilized on polystyrene Petri dishes. While these experiments were successful, the peptides identified failed to reach consensus sequences (*13*). We reasoned that this might be due to insufficient enrichment during phage display screening, which could be easily addressed by increasing the number of biopanning in the phage display experiment. The manufacturer's instructions advise against such modification because of the possibility of potential contaminations. However, by exercising the usual care and applying sterile techniques, including using aerosol-resistant tips, contaminations can be prevented. Throughout our studies, no contamination occurred when the number of panning was increased from the recommended three to five.

In our initial phage display experiment, which aimed to identify peptides bound to LPS-O55, we used the conditions suggested by the manufacturer except for the number of pannings (for details see Materials and Methods). After five rounds of biopanning with LPS-O55, 10 phage clones were randomly selected and subjected to DNA sequencing. Out of 10 clones sequenced, three peptides encoded by more than two clones were found (Table 1). Specifically, peptide NIMRNTW was encoded by four phage clones, peptide AVPRASF by four other clones, and peptide LGSYNNA by two clones (Table 1). Similar results were obtained with O111 and *Salmonella* LPS, though less convergent than that with O55 (data not shown). Presumably, the conditions of phage display could be further modified to improve the enrichment.

In our subsequent experiments with LPS-O55, similar enrichment was achieved with five biopannings (other modifications were introduced, below for details). Out of six phage clones sequenced, two unique peptides were identified: peptides ASFPPAF and SSHTISF, encoded by four and two clones, respectively (Table 1).

The success in obtaining peptides with consensus sequences suggests that enrichment of binding phage clones could be obtained by simply increasing the number of pannings.

Optimizing ELISA Conditions. Typically, after the phage display experiment, the binding specificity and affinity of the phage clones are evaluated with ELISA, as this provides a quick confirmation before applying elaborate procedures. The ELISA

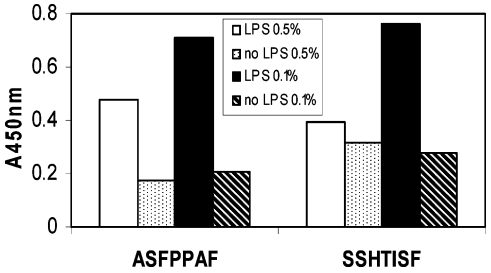


Figure 3. Comparison of ELISA washing conditions with clones selected from subtractive biopanning on LPS-O55.

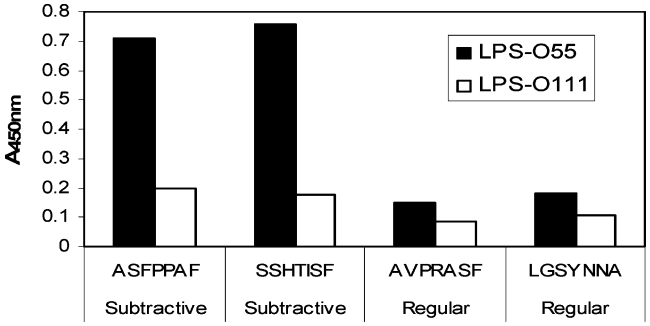


Figure 4. Comparison of phage binding properties between regular and subtractive biopanning on LPS-O55 (■) and LPS-O111 (□).

is based upon an HRP conjugated monoclonal antibody, which recognizes the major coat protein of bacteriophage M13. When phages displaying a LPS-binding peptide are allowed to interact with the ligand (LPS) on a coated plate, the peptide binds to the coated LPS, and thereby the phages retain and are detected with the antibody (Materials and Methods). Many variables influence the final readout. To achieve good signal-to-noise ratio, we carefully studied the effects of different concentrations of BSA in the blocking solution, washing time, incubation time, temperature, and surfactant concentrations of the washing buffer. M13 derived phages tend to stick to unblocked plastic wells, giving high noise levels. Therefore, it is necessary to choose washing conditions to reduce unspecific bindings. The surfactant concentration in the washing buffer is particularly important to the signal-to-noise ratio. A 0.1% concentration of the surfactant PBST (Tween-20) was found to be optimal, giving satisfactory signal-to-noise ratio (Figure 3).

Improving Binding Specificity and Strength by Subtractive Panning. Two peptides obtained from regular panning (Figure 2) were analyzed with the ELISA. The phage clones selected with LPS-O55 were amplified, purified, and tested for interaction with LPSs from *E. coli* O55 and O111 strains. These phages gave a slightly higher signal with LPS-O55 than with LPS-O111, indicating a weak binding preference to O55 (Figure 4). Despite its reproducibility, the signal differences between these two LPSs were within the error range of the experiment, so the binding preference to LPS-O55 was not conclusive. The binding strength, reflected by the OD_{450}, was also low, indicating the binding was of a low affinity nature.

We hypothesized that the binding specificity could be improved if we modified the panning procedure so that it selects against an undesired target. This procedure is referred as subtractive panning. We used LPS-O55 (desired target) and LPS-O111 (undesired target) to test this idea. The goal was to select peptides that bind to LPS-O55 but not LPS-O111. In the subtractive panning, the phage library was first incubated with LPS-O111, upon which the unbound portion was used to select phage clones for binding to LPS-O55. This subtractive process was repeated in each round of panning to ensure no LPS-O111

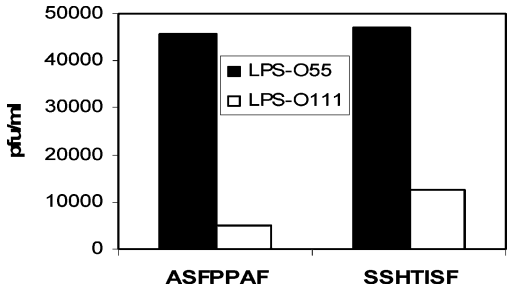


Figure 5. Reactivity of selected clones from subtractive biopanning on LPS-O55 (■) and LPS-O111 (□).

binding clones would be selected (details in Materials and Methods section). In addition, we modified the panning procedure by using a washing buffer with a higher surfactant concentration (1% TBST versus 0.5%) and reduced the binding time (40 min versus 60 min; details in Figure 2). We expect that these modifications will allow the selection of peptides with increased binding strengths.

At the end of the fifth round of panning with this modified procedure, six phage clones were analyzed to deduce the amino acid sequences (Table 1). The binding strength and specificity were analyzed with ELISA. Figure 4 shows a comparison of bindings between peptides obtained using regular and subtractive pannings. The signals with the peptide from the subtractive biopanning were much stronger than those from regular panning, indicating that the binding strength was improved through the modifications in the panning procedure. In addition, the two peptides from subtractive panning bind more strongly to LPS-O55 than to LPS-O111 and the differences in signal were greater than 3.5, whereas the signal ratios of peptides obtained from regular panning were less than 2, suggesting an improvement on the specificity through subtractive panning. To further confirm the binding specificity of the two selected peptide sequences, the numbers of bound phages were quantified. Figure 5 demonstrates that both clones were preferentially bound to LPS-O55. In the case of peptide ASFPPAF, there were nine times more phages bound to LPS-O55 than to LPS-O111. In the case of peptide SSHTISF, there were four times more to LPS-O55 than LPS-O111. These numbers were much higher than those previously reported, which were in the range of 1.6−2.7 (*10*). Therefore, the specificity of binding was significantly improved by using subtractive panning. This points to a general strategy to improve binding by using a dual selection process in the phage display experiment, simultaneously selecting for a desired target and against an undesired target. Conceivably, by including other LPSs in the subtractive panning as undesired targets, we could obtain peptides that are unique in that they only bind the desired target. A potential application of this method is selection of peptides that bind to LPSs from pathogenic strains but not to LPSs from benign O-antigen carriers. The peptides can then be used as molecular recognition elements for detection and subtyping of pathogens. Further studies are needed to characterize the binding strength and specificity by using synthetic peptides with sequences identified in this study.

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