Landscape phage probes for *Salmonella typhimurium*


*Department of Pathobiology, Auburn University, 252 Greene Hall, College of Veterinary Medicine, Auburn, AL 36849, United States*

*Department of Biological Sciences, Auburn University, 101 Life Sciences Building, Auburn, AL 36849, United States*

*Department of Anatomy, Physiology and Pharmacology, Auburn University, 109 Greene Hall, College of Veterinary Medicine, Auburn, AL 36849, United States*

*Department of Mechanical Engineering, Auburn University, 201 Ross Hall, Auburn, AL 36849, United States*

Received 9 September 2004; received in revised form 17 February 2005; accepted 23 February 2005

Available online 11 May 2005

**Abstract**

We selected from landscape phage library probes that bind preferentially *Salmonella typhimurium* cells compared with other *Enterobacteriaceae*. The specificity of the phage probes for *S. typhimurium* was analyzed by the phage-capture test, the enzyme-linked immunosorbent assay (ELISA), and the precipitation test. Interaction of representative probes with *S. typhimurium* was characterized by fluorescence-activated cell sorting (FACS), and fluorescent, optical and electron microscopy. The results show that the landscape phage library is a rich source of specific and robust probes for *S. typhimurium* suitable for long-term use in continuous monitoring devices and biosorbents.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** *Salmonella typhimurium*; Phage display; Landscape phage; Biosorbent; Probe; Detection; Monitoring

---

**1. Introduction**

Screening of potential molecular probes for bacterial identification has long been a central tenet of bacteriology, dating back nearly 300 years to Von Leeuwenhoek, who first attempted visualization of bacteria using naturally colored agents such as beet juice (Wainwright and Lederberg, 1992). Later, Koch developed a heat-mediated methylene blue staining process for the identification of tubercle bacillus, while Hans Christian Gram developed a procedure for the differentiation of bacteria based on cell wall...
components staining (Donnelly, 1962) that is still widely used today. Since then, numerous molecular recognition methods have been elaborated for detection of bacteria, most attempting to incorporate speed and simplicity (Madonna et al., 2001; Mansfield and Forsythe, 2000; Meckes and MacDonald, 2003), as reviewed in Petrenko and Sorokulova (2004). Simplified, rapid identification is especially important for pathogenic food-borne bacteria. Among these, Salmonella typhimurium (S. typhimurium) ranks as a leading cause of bacterial food-borne illnesses in the United States (Campbell et al., 2001).

Classical S. typhimurium identification is based on amplification of cells on selective media (Dusch and Altwegg, 1995). However, this technique may take as many as 4–5 days to confirm identity. More advanced methods for routine S. typhimurium detection include polymerase chain reaction (PCR) (Kumar et al., 2003; Peng and Shelef, 2001; Tseng et al., 1997), DNA hybridization (Meckes and MacDonald, 2003; Miyamoto et al., 1998; Nastasi et al., 1999), enzyme-linked immunosorbent assay (ELISA) (Tapchaisri et al., 1999), and biosensor-based assays (Kramer and Lim, 2004; Muhammad-Tahir and Alocilja, 2003; Olsen et al., 2003; Taitt et al., 2004). These modern methods offer many potential advantages for the rapid detection of microbial and viral pathogens in environmental samples and food in comparison with traditional long-term culture methods. Despite these advantages, their applications for real time detection and environmental monitoring remain in developmental stages with significant methodological hurdles. For example, widespread use of PCR in environmental microbiology has been limited by high sample volumes compared to miniscule amplification reaction volumes and uncontrolled organic impurities and environmental inhabitants inhibiting enzymatic reactions (Lantz et al., 2000). Thus, the uses of many rapid detection techniques could be expanded if the pathogens were separated, concentrated, and purified from the sample matrix before detection reactions (Benoit and Donahue, 2003; Stevens and Jaykus, 2004). Recombinant phage containing luciferase-encoding gene was used recently for the detection of Salmonella in milk using HPLC. The same phage was utilized (Bennett et al., 1997) as a biosorbent probe for separation, concentration, and detection of S. enteritidis in food.

Bacteriophages (phages) are naturally evolved molecular probes for bacteria. They are intrinsically specific to host bacteria and survive in severe environmental conditions (Bennett et al., 1997; He and Pan, 1992). There were numerous attempts to use phages as diagnostic probes for Salmonella. In the early 80s, Hirsh and Martin (1983a,b) utilized phage F01 for the detection of Salmonella in milk using HPLC. The same phage was utilized (Bennett et al., 1997) as a biosorbent probe for separation, concentration, and detection of S. enteritidis in food. Recombinant phage containing luciferase-encoding gene was used recently for the detection of Salmonella in milk using HPLC. The same phage was utilized (Bennett et al., 1997) as a biosorbent probe for separation, concentration, and detection of S. enteritidis in food.

Phage display technique is a new approach for development of phage-derived diagnostic probes (reviewed by Petrenko and Sorokulova, 2004; Petrenko and Vodyanoy, 2003; Smith and Petrenko, 1997)). Our studies of landscape phages – recombinant filamentous phages displaying 4000 foreign random peptides on their surface – showed that these nonlytic secreting viruses of Escherichia coli are well suited for obtaining durable, specific diagnostic
probes, and biosorbents for various analytes (Petrenko et al., 1996). The probes are selected from the phage display libraries, which are constructed by a genetic modification of the filamentous phage fd (Smith and Petrenko, 1997). The outer coat of the phage is dominated by multiple copies of the major coat protein pVIII, which forms a tube encasing the viral DNA and comprises 87% of the phage mass (Webster, 2001). To create a landscape phage display library, degenerate synthetic oligonucleotides are spliced in-frame into the pVIII protein gene, so that the “guest” peptides encoded by the degenerate oligonucleotides are fused to the coat protein and thereby displayed as diverse “organic landscapes” on the surface of the virions (Petrenko et al., 1996, 2002). A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library, f8/8, used in this work contains random octapeptides fused to all 4000 copies of the major coat protein (Petrenko et al., 1996).

Landscape phages have been shown to serve as substitutes for antibodies against various soluble and cell-displayed antigens and receptors (Legendre and Fastrez, 2002; Mount et al., in press; Petrenko and Smith, 2000; Petrenko et al., 2002; Romanov et al., 2001; Samoylova et al., 2003, 2004) including bacterial cells and spores (Brigati et al., in press; Petrenko and Sorokulova, 2004). The phage probes have been used in ELISA and thickness shear mode quartz sensors to detect bacterial and mammalian antigens (Petrenko and Smith, 2000; Petrenko and Vodyanoy, 2003). Phage-derived probes inherit the extreme robustness of wild-type phage (Brigati and Petrenko, unpublished) and allow fabrication of bioselective materials by self-assemble of phage or its composites on metal, mineral, or plastic surfaces. The prospects of phage-derived probes for isolation and detection of biological agents have been illustrated recently (Brigati et al., in press; Petrenko and Smith, 2000; Petrenko and Sorokulova, 2004; Petrenko and Vodyanoy, 2003). In our study, we used a landscape phage library f8/8 (Petrenko et al., 1996) to isolate phage clones displaying peptides capable of specific and strong binding to S. typhimurium [preliminary results of the study were reviewed briefly (Petrenko and Sorokulova, 2004)]. Along with other recently published data (Brigati et al., in press), the results of this work validate the concept of landscape phages as probes for separation, concentration, and detection of biological threat agents.

2. Materials and methods

2.1. Solutions, reagents, and media

Solutions, reagents, preparations, and media are described in Table 1.

2.2. Bacteria and library

S. typhimurium ATCC 13311 was obtained from the American Type Culture Collection, Manassas, VA. E. coli ATCC 25922, Proteus mirabilis ATCC 25933, Enterobacter aerogenes ATCC 13048, Citrobacter freundii ATCC 8090, Shigella flexneri ATCC 12022, Klebsiella pneumoniae ATCC 13882, Yersinia enterocolitica ATCC 9610, Serratia marcescens ATCC 13880, and Pseudomonas aeruginosa ATCC 27853 were acquired from the Auburn University culture collection (Auburn, AL). Cultures were previously confirmed for identity by biochemical, serological, and morphological tests. E. coli K91BlueKan (Yu and Smith, 1996) was used for propagation of phage clones. Stock organisms were maintained in NZY broth containing 20% (v/v) glycerol at −20 °C.

The landscape phage library f8/8, containing about two billion different clones, was previously described (Petrenko et al., 1996, 2002). The library was constructed by replacing amino acids E2, G3, and D4 on every copy of the pVIII coat protein of vector f8-1 (fd-tet derivative) with eight random amino acids.

2.3. Phage growth, purification and titering

The general procedures used for recombinant phage production and analysis, including media and buffers, are detailed in “Phage Display, A Laboratory Manual” (Barbas et al., 2001). Phage were purified by double PEG precipitation as previously described (Smith and Scott, 1993; Yu and Smith, 1996). The total number of viral particles present in phage preparations was determined spectrophotometrically using the formula (Barbas et
al., 2001): virions (vir)/ml = \( (A_{269} \times 6 \times 10^{16}) / \text{number of nucleotides in the phage genome, where } A_{269} \) is absorbance at 269 nm.

For the recombinant phages used in this work (9198 nucleotides), the formula: absorbance unit (AU)\(_{269} = 6.5 \times 10^{12}\) vir/ml was used to determine the concentration of phage particles in a solution (physical titer). The concentration of infective phage particles (biological titer) of a phage solution was determined by infecting starved K91BlueKan cells (Barbas et al., 2001) with the phage, followed by their spreading on a tetracycline-containing agar plate. The recombinant phages carry the gene necessary for tetracycline resistance, allowing only those cells infected by phage to form colonies on the plate. The biological titer of these recombinant phages (expressed as colony forming units/ml or CFU/ml) is typically 20 times lower than the physical titer (vir/ml).

2.4. Selection of bacteria-binding phage clones

In selection procedures A and B, phage particles interacted with bacterial cells S. typhimurium adsorbed to a solid support. In selection procedure C, complexes of phages with S. typhimurium were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Solutions, reagents, preparations, and media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution or Preparation</strong></td>
<td><strong>Description and preparation procedure</strong></td>
</tr>
<tr>
<td>Alkaline phosphatase conjugated goat anti-rabbit antibody IgG (H+L)</td>
<td>Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), diluted 1:5000 in TBS/0.5% Tween</td>
</tr>
<tr>
<td>AP-SA</td>
<td>Alkaline-phosphatase-conjugated streptavidin; Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) 016-050-084; dissolved at a concentration of 500 μg/ml in 5 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM MgCl(_2), 1 mM ZnCl(_2), 50% (v/v) glycerol; stored at 4°C</td>
</tr>
<tr>
<td>AP-SA diluent</td>
<td>1 mg/ml BSA, 0.1% Tween 20, 1 mM MgCl(_2), 0.1 mM ZnCl(_2) in TBS</td>
</tr>
<tr>
<td>Binding buffer</td>
<td>1% BSA, 0.5% Tween in TBS, filter sterilized</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin, Fraction V; Sigma Chemical Co. (Saint Louis, MO) A2153; 50 mg/ml stock is filter-sterilized and stored at 4°C</td>
</tr>
<tr>
<td>BSA covered plates</td>
<td>Add to every Petri dish (35 mm) 1 ml of 1 mg/ml BSA in TBS and keep overnight at 4°C</td>
</tr>
<tr>
<td>DOC lysis buffer</td>
<td>2% sodium deoxycholate, 10mM Tris, 2mM EDTA, pH adjusted to 8.0 with HCl; filter-sterilized</td>
</tr>
<tr>
<td>λ DNA markers</td>
<td>BstEII digest of phage λ DNA: 8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224, and 117 base pairs (bp)</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>Mixture of equal concentrations of dATP, dGTP, dCTP, and dTTP</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>0.1 N HCl, 1 mg/ml BSA, pH adjusted to 2.2 with glycine; made by mixing water, 50 mg/ml BSA, and 0.4 N HCl, pH adjusted to 2.2 with glycine; filter-sterilized and stored at room temperature</td>
</tr>
<tr>
<td>40×GBB</td>
<td>1.68 M Tris, 0.8 M sodium acetate, 72 mM Na(_2)EDTA, pH adjusted to 8.3 with glacial acid</td>
</tr>
<tr>
<td>Incubation buffer for electron microscopy</td>
<td>PBS, 0.1% BSA-C (Aurion, Electron Microscopy Sciences, Hatfield, PA), 20 mM NaN(_3), pH 7.4</td>
</tr>
<tr>
<td>NAP buffer</td>
<td>80 mM NaCl, 50 mM NH(_4)H(_2)PO(_4), pH adjusted to 7.0 with NH(_4)OH; autoclave and store in a refrigerator or at room temperature</td>
</tr>
<tr>
<td>NZY broth</td>
<td>Dissolve 10 g NZ amine A, 5 g yeast extract, and 5 g NaCl in 1 l water; adjust pH to 7.5 with NaOH; autoclave; store at room temperature</td>
</tr>
<tr>
<td>NZY plates</td>
<td>Autoclave 11 g Bacto-agar in 500 ml water in a 2-l polypropylene flask; without cooling, add 500 ml 2× NZY medium at room temperature; add supplements such as tetracycline as required; swirl to mix; pour about 25 ml per 100-mm Petri dish</td>
</tr>
<tr>
<td>PBS</td>
<td>0.15 M NaCl, 5 mM Na(_2)PO(_4), pH 7.0 adjusted with NaOH; autoclave</td>
</tr>
<tr>
<td>PEG/NaCl</td>
<td>100 g PEG 8000, 116.9 g NaCl, 475 ml water; autoclave</td>
</tr>
<tr>
<td>Salmonella O Poly A-1 and Vi Antiserum</td>
<td>Becton-Dickinson and Company (Franklin Lakes, NJ), diluted 1:500 in TBS/0.5% Tween</td>
</tr>
<tr>
<td>p-Nitrophenyl Phosphate tablets (NPP)</td>
<td>Sigma (Saint Louis, MO), 5 mg substrate per tablet</td>
</tr>
<tr>
<td>NPP diluent</td>
<td>For 1 tablet: 5 ml 1M diethanolamine buffer (pH 9.8), 5 μl 1M MgCl(_2)</td>
</tr>
<tr>
<td>TBS</td>
<td>50 mM Tris–HCl (pH 7.5), 0.15 M NaCl; autoclave</td>
</tr>
<tr>
<td>TBS/Tween (washing buffer)</td>
<td>0.5% (v/v) Tween 20 in TBS; filter-sterilized</td>
</tr>
</tbody>
</table>
formed in solution and were separated from unbound phages by precipitation, as described below.

2.4.1. Selection procedure A

*S. typhimurium* was propagated in 20 ml NZY medium in a shaker-incubator (16 h, 200 rpm, 37 °C), precipitated (5500 rpm, 15 min, Allegra 21R Centrifuge, Beckman Coulter, rotor S4180), washed with 20 ml PBS, and resuspended in 4 ml PBS. A portion of the suspension (1 ml) was spread onto an empty 35 mm Petri dish and dried overnight at 37 °C. The dish was blocked with 0.1% BSA in TBS (1 h RT) then washed 3 times with 1 ml TBS/0.1% Tween. The f8/8 phage library (10^{11} virions in 400 μl TBS, 0.1% BSA) was added to the dish and incubated 1 h at room temperature (RT). Nonbound phage particles were removed, and the dish was washed 6 times with 1 ml of TBS/0.1% Tween. Phages bound to *S. typhimurium* were eluted with 400 μl of elution buffer (10 min RT). The eluate was transferred to a microcentrifuge tube and neutralized with 75 μl 1M Tris (pH 9.1). The dish was washed with 0.5 ml TBS, the washing was combined with the eluate, and concentrated by centrifugation in a Centricon unit (100 kD, Millipore Corp., Bedford, MA) to ~100 μl. Remaining cell-associated phages in the dish were recovered by lysing cells with the DOC lysis buffer (250 μl, 30 min at RT). The phages from acid elution and DOC lysate fractions were propagated separately in 20 ml NZY, containing 20 μg/ml tetracycline and 100 μg/ml kanamycin, purified by double PEG precipitation (Barbas et al., 2001) and suspended in 200 μl of TBS buffer. During the second round of selection, portions of the amplified phage preparations (100 μl) were added to two bacteria-coated dishes (prepared and blocked with BSA as described above), which were incubated 1 h at RT. The dish with phages originated from the elution fraction was washed 6 times with 1 ml of TBS/0.1% Tween, and bound phages were recovered with acid elution buffer, as described above. Another dish, with the phages originated from the DOC lysis fraction, was also treated with elution buffer, washed once with 1 ml of TBS, and the remaining phages were extracted with the DOC lysis buffer. Phages from the acid elution fraction (second dish) were amplified and purified as in the first round. Likewise, the phages selected and amplified in the previous rounds were then used as an input in each subsequent round. Following the fifth round of selection, individual phage clones were amplified and sequenced to determine the amino acid sequences of the displayed peptides, as described in Section 2.5 below.

2.4.2. Selection procedure B

Proceeding the selection of *Salmonella*-binding phages, the library was depleted of phages reacting with plastic and immobilized BSA, as follows. The primary f8/8 phage library (10^{11} virions in 400 μl of TBS/0.5% Tween/0.1% BSA) was added to an empty 35 mm plastic Petri dish and incubated 1 h at RT on the rocker. The phage suspension was decanted, transferred to another 35 mm plastic Petri dish previously blocked with 0.1% BSA in TBS (16 h at 4 °C), and incubated for 1 h at RT. The depleted library was recovered from the dish and transferred to a third 35 mm plastic Petri dish coated with *S. typhimurium* and blocked with 0.1% BSA in TBS (1 h RT), as previously outlined in Section 2.4.1. Four proceeding rounds were performed as previously outlined (Section 2.4.1.), except washing with 0.5% Tween in TBS.

2.4.3. Selection procedure C

To avoid selection of self-precipitating phage clones, the library (10^{11} virions in 400 μl of TBS) was first incubated at 70 °C for 10 min followed by adding 40 μl of 5% Tween-20. The phage suspension was centrifuged at 13,000 rpm 15 min [here and later for centrifugation in microtubes, we used Microfuge 18 Centrifuge (Beckman Coulter, Fullerton, CA)] before adding 400 μl *Salmonella* cells (OD_{_620} 0.8). After incubation of the mixture of *S. typhimurium* with phages for 1 h, complexes of bacteria with bound phage particles were precipitated (3500 rpm, 10 min) and washed 10 times by centrifugation (3500 rpm, 10 min) with 1 ml TBS/0.5% Tween. Following the final wash, the precipitate of cell-bound phages was suspended in 400 μl elution buffer and incubated for 10 min at RT. Cells were precipitated (3500 rpm, 10 min), the supernatant (eluate) was recovered and neutralized with 75 μl 1 M Tris (pH 9.1), and the pellet was
solubilized in 250 µl DOC lysis buffer (30 min, RT, roller). Phage from both fractions (eluate and lysate) was amplified separately in *E. coli* K91BlueKan and used as the input for two proceeding rounds of selection, carried out as previously described (cf. Section 2.4.1). Following the third round of selection, individual phage clones were amplified and sequenced to determine the amino acid sequences of the displayed peptides, as described in Section 2.5 below.

### 2.5. PCR and DNA sequencing

Sources of phage DNAs for PCR and sequencing were colonies of infected bacteria and solutions of purified phages. Primers f8s-20 (5′-CAAAGCCTCCTCGTAGCCGTTG-3′) and f8as-20 (5′-CATTCCACA-GACAGCCCTCA-3′) were obtained from Integrated DNA technologies, Inc., Coralville, IA.

A sample (1 µl) of the suspended colony or phage diluted 1:100 in water was added to the PCR reaction tube, containing 24 µl reagents for PCR: 10 mM Mg free reaction buffer (Promega, Medison, WI, USA) (2.5 volumes); 25 mM MgCl₂ (Promega) (2 volumes); Taq DNA polymerase (5 units/µl (Promega) (0.04 volumes); 2.5 mM dNTPs (2 volumes); primer f8s-20 (10 pmol/µl) (0.3 volumes); primer f8as-20 (10 pmol/µl) (0.3 volumes); doubly-distilled filter-sterilized water (16.9 volumes). The PCR was carried out using GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) under the following cycling conditions: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 10 s, 46 °C for 20 s, 72 °C for 45 s; and one cycle at 72 °C for 4 min. PCR products (3 µl) were analyzed by agarose gel-electrophoresis (0.8% wt/vol agarose, Sigma, St. Louis, Mo, USA) in 4× TBE buffer. Gel was stained with SYBR Green I nucleic acid gel stain (Cambrex BioScience Rockland, Rockland, ME, USA), illuminated on the Dark Reader (Clare Chemical Research, Denver CO), and DNA bands were visualized using a Kodak EDAS 290 imaging system (Eastman Kodak Company, Rochester, NY, USA). PCR products were purified using QIAquick PCR purification Kit (QIAGEN Inc., Valencia, CA) and sequenced at the Auburn University Genomic and Sequencing Laboratory (Auburn, AL) using primer f8s-20.

#### 2.6. Co-precipitation assay

Phage suspension (300 µl, 10⁹ CFU/ml in TBS) heated at 70 °C for 10 min was mixed with 7.5 µl of 20% Tween-20 and centrifuged at 13,000 rpm for 15 min to precipitate phage aggregates. Supernatant (100 µl) was added to a suspension of bacterial cells (400 µl, OD₆₂₀ 0.8, TBS/0.5% Tween) and incubated 1 h at RT on a 360° rotator (Barnstead International, Iowa, Model 400110) to affect specific phage-cell binding. Cells were precipitated (3500 rpm for 10 min), washed five times with 1 ml TBS/0.5% Tween, lysed in 50 µl DOC lysis buffer (30 min at RT), and diluted with 450 µl TBS. Negative controls (possessing no phage) were prepared by mixing 100 µl TBS/0.5% Tween with 400 µl aliquots of bacterial suspension. As well, a negative control (possessing no bacteria) containing only 100 µl of phage and 400 µl TBS/0.5% Tween was prepared. Phage titers were determined as described in Section 2.3. A normalized, mean recovery (output/input × 100) percentage and deviation was calculated for three duplicates.

Formation of the complexes between phages and bacteria was described through known binding equations (Connors, 1987) with some special considerations. We assumed that one or more binding sites on the phage can be involved in binding with one or more identical complementary cell surface receptors. Since the phage-complementary surface receptors on *S. typhimurium* cells are unknown, as well as a stoichiometry of their interaction with the individual phage outer coat peptides, we described the reaction between phage particles (*P*) and cell receptors (*S*) by the equation:

\[nP + S \rightarrow SP_n\]  

where, *n* is the Hill coefficient, which may correspond to the number of phage particles bound to a single cell receptor; the concentration of phage in this assay is variable, and the total concentration of the cells is kept constant (≈10⁸ cells/ml).

The overall binding constant (*Kₚ*) for the reaction (1) can be defined by the law of mass action (Connors, 1987):

\[K_p = \frac{[SP_n]}{([S][P]^n)}\]
The total number of cell receptors (Cs) is composed of free receptors and those bound with phage:

\[
Cs = [S] + [SP_n].
\]  

(3)

Combining Eqs. (2) and (3) yields that fraction of cell receptors complexes with phage:

\[
Y = [SP_n]/Cs = K_b [P]^n/(1 + K_b [P]^n).
\]  

(4)

The ratio of receptors bound with phage to free receptors can therefore be defined by the equation:

\[
\]  

(5)

Taking the logarithm of both sides yields:

\[
\log(Y/(1 - Y)) = \log(K_b) + n \log[P]
\]  

(6)

A Hill plot of \(\log(Y/(1 - Y))\) versus \(\log[P]\) allows an estimate of the Hill coefficient \((n)\) from the slope, and association binding constant \((K_b)\) from the ordinate intercept.

The portion of the cell receptors bound with phage \((Y)\) was determined from the experimental binding isotherm as:

\[
Y = Pb/P_{max}
\]  

(7)

where Pb is a number in infectious phage particles eluted from bacterial cells; and \(P_{max}\) is a maximal saturating number of the bound infectious phage particles, determined by sigmoidal approximation of the binding curve.

The dissociation constant can be calculated from \(K_b\) as follows:

\[
K_d = 1/K_b.
\]  

(8)

The \(K_d\) apparent is calculated by taking the Hill coefficient into account:

\[
K_d(\text{apparent}) = [K_d]^{1/n}.
\]  

(9)

2.7. Fluorescently labeled phage

Phage VTPPTQHQ (here and later the names of selected phages correspond to the sequences of the phage-harbored foreign peptides) \((1.2 \times 10^{14} \text{ phage particles})\) was fluorescently labeled using Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Eugene, OR) as recommended by the manufacturer and isolated by double PEG-precipitation. Phage yield \((9.8 \times 10^{13} \text{ vir})\) and degree of labeling \((\sim 300 \text{ dye residues per phage})\) were determined by measuring the phage absorbance at 269 and 494 nm (dye absorption maximum) as recommended by the manufacturer.

2.8. Binding of fluorescently labeled phage to \(S.\ typhimurium\)

Gradually diluted solutions of fluorescently labeled phage \((100 \mu l, \text{subsection 2.7.})\) were mixed with suspensions of \(400 \mu l S.\ typhimurium\) \((\text{OD}_{620} 0.8 \text{ in TBS/0.5\% Tween})\) and rotated \(1 \text{ h at RT. The samples were centrifuged (3500 rpm for 10 min), decanted, and the pellets were washed twice with TBS/0.5\% Tween. Following the final wash, the pellets were resuspended in PBS and analyzed by FACS (Coulter EPICS Elite Flow Cytometer, Beckman Coulter, Fullerton, CA) and fluorescence microscopy (Nikon Eclipse E800, Melville, NY).}

2.9. Electron microscopy

Phage VTPPTQHQ was biotinylated as described below. A mixture of \(70 \mu l\) of EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology Inc., Rockford, IL) \((1 \text{ mg/ml in 2 mM NaOAc pH 6})\) and \(1 \text{ ml of the phage in PBS (6.7 \times 10^{13} \text{ vir/ml}}\) was incubated on the roller for \(2 \text{ h at room temperature. The reaction was quenched with 0.27 ml of 1M Tris–HCl (pH 8.9) for 1 h at RT and the preparation was dialyzed in Slide-A-Lyzer 10K Dialysis cassette (PIERC\E, Rock ford, IL) against 4 \times 1 \text{l of TBS buffer at RT. The level of phage biotinylation (478 biotin residues per phage particle) was measured using EZ’™ Biotin Quantitation Kit (Pierce Biotechnology Inc., Rock ford, IL) according to the manufacturer’s procedure. Suspension of the complexes of the biotinylated phage with S. typhimurium (10 \mu l), obtained as described in Section 2.6, was loaded onto formvar/carbon-coated grid and was incubated for 20 min at RT. Excess fluid was removed by absorption to a filter paper and the grid was incubated (30 min RT) with 10 \mu l EM Streptavidin-conjugated gold particles (10 nm, BB International, Cardiff, UK), diluted 1:30 with incubation buffer. The grid was washed with incubation buffer, negatively stained (0.2\% phosphotungstic acid for 5 min), and examined using Philips 301 TEM at 60 kV).
2.10. Phage capture ELISA

Wells of a 96-well ELISA dish were coated overnight at 37 °C with 100 µl suspensions of *S. typhimurium* (OD₆₂₀ 1.0) in PBS; washed five times with TBS/0.5% Tween on an automatic plate washer; blocked with 0.1% BSA in TBS and incubated for 1 h at RT; washed again; reacted with phage clones (5 × 10¹⁰ vir in 50 µl of binding buffer for 1 h at RT); washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 µg/ml) for 1 h at RT; washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 µg/ml) for 1 h at RT; washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 µg/ml) for 1 h at RT; washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 µg/ml) for 1 h at RT; washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 µg/ml) for 1 h at RT; washed again. Wells were then filled with 90 µl of NPP substrate and read on a kinetic plate reader as previously described (Yu and Smith, 1996). The slope of color development was measured as a change in optical density per 1000 min (mOD/min). Wild-type vector f8-5 (Petrenko et al., 2002) served as a negative control for evaluation of nonspecific background binding.

2.11. Salmonella capture ELISA

Wells of a 96-well ELISA dish were coated overnight at 4 °C with 2.7 × 10¹⁰ virions in 55 µl TBS; washed five times with TBS/0.5% Tween on a plate washer; blocked with 1% BSA in TBS and incubated 1 h at RT, washed and reacted with approximately 5.0 × 10⁷ cells *S. typhimurium* in 50 µl TBS/0.05% Tween (OD₆₂₀ 0.8 diluted 1:20) for 2 h RT; washed again on a plate washer; reacted for 1 h at RT with 45 µl *Salmonella* O Poly-1 antiserum (Becton, Dickinson and Company, Franklin Lakes, NJ) (diluted 1:500 in TBS/0.5% Tween); washed again on washer; reacted for 1 h at RT with 40 µl AP-conjugated goat anti-rabbit antibodies (diluted 1:5000 in TBS/0.5% Tween (Jackson ImmunoResearch Laboratories Inc., West Grove, PA)); washed again; and developed with NPP substrate as previously described in Section 2.10 above. The slope of color development was measured as a change in optical density per 1000 min (mOD/min). Wild-type vector f8-5 served as a negative control for evaluation of nonspecific background binding.

2.12. Binding of Salmonella to the gold-immobilized phage

A gold strip (5 × 3 × 0.05 mm) was rotated for 1 h at RT with phage suspension (5 × 10¹¹ vir/ml in PBS), washed 5 times with PBS to remove unbound phage and soaked in suspension of *S. typhimurium* diluted in PBS (OD₆₂₀ 1.0) on rotator for 3 h at RT. Subsequently, the strip was washed with doubly distilled filter-sterilized water and analyzed by optical microscopy (Mitutoyo America Corporation, Aurora IL, or Nikon, Marietta GA) and scanning electron microscopy (Zeiss DSM 940 Scanning Electron Microscope, Thornwood, NY). The samples for scanning electron microscopy (SEM) were prepared by exposure to OsO₄ for an hour, air drying for 30 min, and sputter coating with gold according to the standard SEM procedures.

3. Results

3.1. Selection of phage clones that bind to *S. typhimurium*

The source of probes for *S. typhimurium* was the landscape library f8/8, which contains random octa-peptides fused to all 4000 copies of the major coat protein of fd-tet (Petrenko et al., 1996). We explored three differing selection procedures outlined in Table 2. In selection procedure A, a portion of the library

---

**Table 2**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Separation of bacteria/phage complexes</th>
<th>Blocking, BSA (%)</th>
<th>Library depletion</th>
<th>Wash, Tween (%)</th>
<th>Rounds of selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Immobilization</td>
<td>0.1</td>
<td>NO</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>Immobilization</td>
<td>0.1</td>
<td>Plastic, BSA-coated plastic</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>Precipitation</td>
<td>0</td>
<td>Centrifugation</td>
<td>0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

---
(2 × 10^9 clones, 10^{11} virions) in TBS/0.1% BSA was added to 35 mm Petri dish coated with immobilized Salmonella. Following incubation, nonbinding phage were washed away, and specific cell-associated phage was eluted with mild acid. Residual cell-bound phage virions were recovered by lysing Salmonella cells with DOC buffer (Ivanenkov et al., 1999). The two fractions (eluate and lysate) containing cell-specific phage were amplified separately in E. coli K91Blue-Kan host strain in the presence of kanamycin—antibiotic that destroys any Salmonella cells present in the fractions, but spares the host (kan+) cells. Sub-libraries enriched for Salmonella-binders were used in subsequent rounds of selection. In procedure B, we used more stringent conditions for selection: the library was depleted against plastic and BSA, and incubated with bacteria in the 0.5% Tween-20-containing buffer.

Taking into account that Salmonella can display different receptors under differing environmental conditions (Gawande and Bhagwat, 2002), we exploited also procedure C, in which phage clones bound to suspended Salmonella cells were separated by centrifugation. To avoid selection of self-aggregating and precipitating phage clones, the library was first heated at 70°C and centrifuged and then rotated with a suspension of S. typhimurium. Two fractions of cell-associated phages isolated by acid elution followed by lysis of cells were amplified separately in E. coli strain K91BlueKan and used for subsequent rounds of selection. During the selection, the relative portion of Salmonella-associated phage in amplified phage fractions increased from one round to another (Table 3) indicating productive selection of bacterial binders. Following the 5th (A), 4th (B), or 3rd (C) rounds, individual phage clones were analyzed by PCR and DNA sequencing and propagated for further screening.

Identified phage borne peptides can be grouped into at least five homologous families (Table 4). Peptides isolated in procedures A and B from acid fractions demonstrate striking similarities and form the family I with a leading motif VT/SP, followed by dominant P, frequent T/S, frequent T/S/Q, and frequent H. Dominant phage clone VSSNQAPP in Selection C (for simplicity, phage clones are designated by the peptide sequences they bear) is noticeably similar to that of one phage clone VSPQSAPP from selection A and may belong to the family I or may form a discrete family. Another dominant phage DRSPSSPT, eluted from suspended bacteria by acid, belongs to the family III formed by phages eluted from immobilized bacteria with DOC buffer in procedure A. Two phages VSPPSNPS and VTPPSQHA found in DOC fraction in procedure A, and phage VTPPQQGS, found in DOC fraction in procedure B, belong to the family I and probably originated from the corresponding acid fractions that can contaminate the DOC fractions. Thus, analysis of phage sequences shows that library depletion and more stringent conditions of incubation

<table>
<thead>
<tr>
<th>Procedure²</th>
<th>Yield (output/input), %/round of selection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Selection A</td>
<td>Acid fraction</td>
</tr>
<tr>
<td></td>
<td>DOC fraction</td>
</tr>
<tr>
<td>Selection B</td>
<td>Acid fraction</td>
</tr>
<tr>
<td></td>
<td>DOC fraction</td>
</tr>
<tr>
<td>Selection C</td>
<td>Acid fraction</td>
</tr>
<tr>
<td></td>
<td>DOC fraction</td>
</tr>
</tbody>
</table>

² Three different protocols were used for selection of the Salmonella-binding phages: A, B, and C, as described in Materials and methods, Sections 2.4.1, 2.4.2, and 2.4.3.
and wash in the procedure B versus procedure A influenced essentially composition of phage in both the acid and DOC fractions. Furthermore, change of mode of separation for phage-bacterial complexes in the procedure C dramatically effected the composition of the phages isolated from the acid fraction and did not permit isolation of any phage from the lysis fraction. This may imply that substrate-immobilized bacteria, versus bacteria in suspension, display different receptors, or that multivalent binding of phage to bacteria in suspension occurs less readily than to the bacteria adsorbed to a plastic matrix.

### 3.2. Specificity of phage binding to S. typhimurium

We define specificity as the ability of a landscape phage, possessing specific peptide sequences, to interact with a selector bacterium displaying complementary surface receptors. To determine specificity, we compared the binding of the selected phage clones...
with that of wild-type phage (f8-5, see Materials and methods) and nonrelated recombinant phage from the f8’8mer library.

For screening of prospective phage probes, the relative binding of the isolated phage clones to S. typhimurium was estimated by phage-capture ELISA and a bacteria-capture ELISA. In the phage-capture assay, selected phage clones were added to the wells of a microtiter plate coated with S. typhimurium cells. Following incubation to effect specific-binding between cell and phage, nonbinding phage particles were washed away and bound phages were revealed using biotinylated anti-phage antibodies. As shown in Table 4, selected clones bind to S. typhimurium to a much greater degree, in comparison to wild-type phage. In the bacteria-capture ELISA, wells of a microtiter plate were coated with phage then incubated with S. typhimurium. Binding of bacteria to immobilized phage was detected with rabbit anti-S. typhimurium antibodies, followed by reaction with alkaline phosphatase-labeled goat anti-rabbit antibodies and p-nitrophenyl-phosphate. The majority of the isolated phage clones bound to Salmonella at a higher degree in comparison to wild-type phage. Some clones bound strongly to S. typhimurium in both assays, while other clones gave inconsistent results between the two assays. This is not completely unexpected because in the bacteria-capture ELISA, phage is fixed to the plate and cells are captured from solution, while in the phage-capture assay, cells are fixed to the plate and phages are bound from solution. Thus, in these, tests phage can adopt different conformations allowing monovalent or multivalent interactions with the bacterial receptors. This has been demonstrated in binding experiments in which β-galactosidase from E. coli served as a model multivalent analyte (Petrenko and Vodyanoy, 2003). Results of bacteria- and phage-capture ELISAs may differ also because the anti-fd phage antibodies used for revealing of cell-bound phage in the phage-capture assay can bind unequally with different recombinant phage clones.

Representative clones demonstrating ELISA signals higher than control wild-type phage were characterized by a precipitation assay to confirm their specificity for Salmonella. This assay, previously described for the analysis of phage binding to zoospores and bacterial spores (Bishop-Hurley et al., 2002; Knurr et al., 2003), was optimized to avoid aggregation and self-precipitation of phage (Brigati et al., in press). Using this assay, we found that yields of Salmonella-bound phages were 12,000–22,000 times higher than the yield of the control wild-type phage, indicating very high specificity of the selection procedure.

Phage VTPPTQHQ demonstrated the highest binding to the bacterium in precipitation assay and was studied in more detail to estimate affinity and selectivity of its binding to Salmonella. Dose-dependent binding of phage to cells in solution was observed in phage concentrations ranging from 10^1 to 10^{12} CFU/ml (Fig. 1A) with maximum yield of bound phage ~ 5 × 10^7 CFU (~ 10^9 vir), comparable or exceeding the number of bacterial cells involved in the complex formation (~ 10^8). Indeed, the TEM images of bacteria-complexed phage clearly show that more than one phage particle can bind to one bacterial cell (Petrenko and Sorokulova, 2004). The highest recovery of bound phage (15%), measured as a ratio of output to input phage, was obtained when equal or surpassing amounts of bacteria were involved in the complex formation (10^6–10^8 CFU/ml phage versus ≈10^8 CFU/ml S. typhimurium) (Fig. 1B). The K_d of the complex calculated from the Hill plot (Fig. 1C) is 4.77 × 10^7 CFU/ml (K_{d(apparent)}=3.18 × 10^9 CFU/ml), corresponding to 1.6 pM of phage, or 6.3 nM of the phage-borne peptide (K_{d(apparent)}=106 pM phage and 422 nM peptide). The extraordinary high affinity of phage binding may be attributed to an avidity effect of the multivalent interaction of phage, carrying 4000 binding peptides, with multiple bacterial surface receptors. The multivalent interaction of phage with cells appears to be confirmed by TEM data (Petrenko and Sorokulova, 2004). Hill coefficient (n = 0.81) estimated from Hill plot (Fig. 1C) also indicates that one phage particle may bind to more then one bacterial cell receptor. The affinity data of phage-cell interaction, however, should be considered with prejudice because we cannot exclude that bacterial cell receptors are not equally competent to phage binding.

Binding of phage VTPPTQHQ to Salmonella was also confirmed by fluorescence-activated cell sorting, and visualized by fluorescent microscopy, transmission, and scanning electron microscopy. The phage was fluorescently labeled by Alexa Fluor 488 (Molecular Probes) with a density of 300 dye molecules per phage. Fluorescently labeled phage (10^9 CFU) was incubated with Salmonella cells at...
room temperature for 1 h and centrifuged at 3500 rpm, as in the precipitation test. The complex of phage with bacteria was detected by FACS (Petrenko and Sorokulova, 2004) and visualized by fluorescence microscopy (not shown here).

We also observed binding of *Salmonella* to the immobilized phage directly, by high power optical microscopy and scanning electron microscopy. In this test, small gold chips were coated with the selected test phage and an unrelated control phage using chemisorptions and/or hydrophobic absorption. The chips were soaked in a suspension of *Salmonella* cells, washed, and analyzed microscopically. Using this test, we observed in real time specific binding of

---

**Fig. 1. Binding of phage VTPPTQHQ to *S. typhimurium* as demonstrated by coprecipitation-titration assay.** (A) Concentration-dependent binding isotherm of phage to *S. typhimurium*. Smooth line is B-spline curve fit to data points. Bars=S.D. (B) Mean values (n=3) of yield (output/input, %) are plotted as a function of mean input concentration of phage VTPPTQHQ. (C) Hill plot of binding isotherm. The ratio of bound and free *S. typhimurium* receptors is shown as a function of phage VTPPTQHQ concentration. Line represents the linear least squares fit of experimental data (R=0.967; S.D.=0.612, p=<0.001). Hill coefficient, n=0.81, and association binding constant (Kₐ=2.1×10⁻⁸ CFU/ml; K₈(apparent)=3.18×10⁹ CFU/ml), were derived from the plot. Explanation of the Hill plot is provided in subsection 2.6.
bacterial cells to the immobilized phage (Fig. 2), and we confirmed this binding by scanning electron microscopy (Fig. 3).

3.3. Selective binding of phage to S. typhimurium

We define selectivity as the ability of a recombinant phage clone to preferentially interact with a selective target, i.e. bacteria, in comparison to other potential targets. Binding of the phage probes with S. typhimurium was compared with their binding to control strains of other gram-negative bacteria, predominately Enterobacteriaceae, chosen for their phylogenetic relatedness to S. typhimurium. In the co-precipitation assay, each organism was incubated with an equal amount of the phage VTPPTQHQ, and bound virions, recovered by lysis of bacteria with DOC buffer, were titrated by infecting E. coli K91BK host cells.

Fig. 2. Interaction of S. typhimurium ATCC 13311 with the phages, attached to the gold surface: (A) nonrelated phage EAGPRSAP; (B) selected phage VTPPTQHQ.

Fig. 3. Study of interaction of S. typhimurium ATCC 13311 with selected phage VTPPTQHQ by scanning electron microscopy: (A) plain gold surface nontreated with phage and bacteria; (B) gold surface, coated with phage and treated with bacteria (the arrow shows an attached Salmonella cell).
Precipitation assay demonstrated 10–1000 times greater binding of phage to S. typhimurium versus the challenge bacteria (Fig. 4).

4. Discussion

Monitoring of food and environmental samples for biological threats, such as S. typhimurium, requires probes that specifically bind biological agents and insure their separation, purification, and detection (Mansfield and Forsythe, 2000; Petrenko and Sorokulova, 2004). These binding probes can be isolated from diverse peptide or antibody libraries using phage display (Petrenko and Vodyanoy, 2003). The prospect of this technology has been proven by the development of probes for various bacterial, viral, and toxic agents (Petrenko and Sorokulova, 2004). While the peptides and antibodies discovered through phage display are valuable probes for laboratory assays, landscape phage probes may be better suited for food and field monitoring, where robust inexpensive probes and biosorbents are needed. These probes may be recruited from diverse libraries of landscape phages originated from the filamentous phage fd (Petrenko and Smith, 2000; Petrenko et al., 1996).

Like the parental phage fd, which is probably the most stable natural nucleoprotein withstanding high temperatures, denaturing agents, organic solvents, mild acids, and alkaline solutions, the phage-derived probes, burdened by thousands of guest peptides, inherit the extreme robustness of the wild-type phage. Furthermore, phage is an inexpensive standard construction material that allows fabrication of bioselective layers by self-assemble of virions or their composites on solid surfaces (Petrenko and Vodyanoy, 2003).

The landscape phage library contains many potential probes for surface markers of S. typhimurium, which can be isolated by single- or multistage selection using immobilized or suspended bacteria as a selector. In this study, we elaborated three different selection procedures, resulting in discovery of five families of bacteria-binding peptides (Table 4). One feature that stands out among these peptides is the high amount of proline, which is known to dominate in some antibacterial peptides (Cudic et al., 2002). Specificity of isolated phage clones for S. typhimurium was demonstrated using two variations of ELISA (phage capture and Salmonella capture) and confirmed by precipitation assay, fluorescence and electron microscopy. We also compared binding of representative clone VTPPTQHQ to S. typhimurium and a panel of select bacteria, primarily Enterobacteriaceae, revealing high selectivity of the phage to the selector bacterial strain. However, greater cross-reactivity was noted with Y. enterocolitica and C. freundii that other challenge bacteria. It was not surprising since we used nonbiased selection conditions allowing phages from the diverse landscape library to interact with any one of numerous surface receptors of S. typhimurium, including those that may be common for the panel strains.

In fact, the strains from the family Enterobacteriaceae are highly similar in their surface structure. The major components of their outer membrane include the lipopolysaccharide (LPS), phospholipids, proteins, and a unique cell surface glycolipid—their common antigen (Rick and Silver, 1996). In gram-negative bacteria, composition of LPS is highly variable between genera, species, and even strains, differing in a number and structure of repeating oligosaccharide units, known as O-antigens or O-
chains, which are linked to lipid A via a genera-specific core polysaccharide. These differences account for serotype specificity; to date, well over 40 major serogroups in Salmonella species, over 170 serotypes in E. coli, greater than 40 in Shigella spp., Citrobacter spp., and Proteus spp., and greater than 10 in Klebsiella spp. have been identified (Weintraub, 2003). LPS has been shown to serve as receptors for some natural strain-specific phages (Romanowska et al., 1976) and may serve as a target for landscape phages. If it is true, then some cross-reactivity of S. typhimurium-targeting phage with C. freundii and Y. enterocolitica may be attributed to identical or near identical monosaccharide and disaccharide O-antigen components, which have been noted between Salmonella and Citrobacter species (Knirel et al., 2002).

Numerous highly specialized, variable major and minor proteins, interlaced among the LPS, may be also plausible targets for landscape phages. They serve common functions among the gram-negatives including selective and nonselective translocation and structural integrity, e.g. murine-lipoprotein and OmpA (Benz and Bauer, 1988), but may vary in primary structure. Major proteins of the gram-negative bacteria in general include beta-barrel OM porins such as OmpF, OmpC, OmpD, PhoE, Oms1, OmpN, NmpC, and OprP, which allow nonspecific, hydrophilic, low molecular weight substances to diffuse across the membrane by size exclusion, and specific porin receptors that bind particular extracellular target substances via molecular recognition such as LamB (maltodextrines, mono- and disaccharides), Tsx (nucleotides), ScrY (saccharose), OprB (phosphate), or OprO (P. aeruginosa-pyrophosphate). Minor proteins include iron transporters such as FhuB and those that function in vitamin uptake.

In our experiments, prior to affinity selection, the phage library was propagated in E. coli cells, which may result in elimination of clones specific for E. coli-surface receptors. Therefore, it is reasonable to hypothesize that the phage-targeted receptor(s) of on the surface of S. typhimurium differ from E. coli receptors. A comparison of OmpC, OmpF, and NmpC porins between the two strains has shown that only half of the proteins are similar or identical (Lee and Schnaitman, 1980). For example, N-acetyl phenylalanine β-naphthyl esterase (ApeE) has been demonstrated in S. typhimurium but not E. coli (Carinato et al., 1998); whereas, OmpA, a phage receptor of E. coli (Osborn and Wu, 1980), has not been demonstrated in S. typhimurium.

Finally, there exist several outer membrane associated proteinaceous structures such as fimbriae, and in some genera, pili and flagella, which are antigenic and highly variable between genera. In particular, fimbriae are hair-like appendages composed of protein subunits called fimbrins, which extend outward from the bacterial surface and may function as an adhesion factor for specific receptors on target cells. Fimbriae are prevalent on S. typhimurium as well as other enteric bacteria; multiple types can be expressed by a single isolate. Assuming that the fimbriae are the source of phage affinity for S. typhimurium, cross-reactivity of the phage with Salmonella and Citrobacter in precipitation assay may be explained by a distinct serological relationship between their Type I fimbriae (Nowotarska and Mulczyk, 1977). Any fimbrial relationship between S. typhimurium and Y. enterocolitica is unknown. Thus, while the outer membrane-associated structures are common to gram-negative bacteria, and in particular the enteric bacteria, the potential structural dissimilarity in the components between genera lends itself to explain the selective binding S. typhimurium exhibited by the isolated landscape phage. Identification of the phage-targeting structures on the surface of bacterial cell would allow a clear explanation of phage specificity and selectivity.

Landscape phages discovered in this work may find application as biosorbents and diagnostic probes for monitoring of Salmonella by various devices in which antibodies or peptides have been used. For example, they may be used for separation and purification of bacteria prior to their identification with polymerase chain reaction, immunoassays, flow cytometry, or other methods. Furthermore, they may find application as biorecognition elements of real-time biosensor devices. We recognize that described probes are not completely ideal for identification of S. typhimurium because they cross-react with another enterobacteria. However, the power of phage display technology allows control of the phage selectivity by engineering the biased selection schemes (Petrenko and Sorokulova, 2004). Thus, cross-reacting clones may be removed from the library by its prior depletion against competing strains, as was previ-
ously demonstrated (Petrenko and Sorokulova, 2004). The depleted library will serve as a reservoir of probes for unique *S. typhimurium* markers. If necessary, the affinity of probes may be also increased by directed molecular evolution, including iterative mutagenesis of phage and selection of the bacterial-binders in more stringent conditions (Petrenko and Sorokulova, 2004). Therefore, it is our contention that performance of the selected phage-derived probes can be gradually enhanced through biased selection strategies and directed molecular evolution. We believe that this new generation of robust and inexpensive phage-derived probes will serve as efficient substitutes for antibodies in separation, concentration, and detection systems employed for food and environmental monitoring.

Acknowledgments

This work was supported by Army Research Office grant # DAAD 19-01-10454 (to VAP), Auburn University Animal Health and Disease Research Funds grant #354 (to VAP), and US Department of Agriculture, Special Research Grants (to BAC). We are grateful to Dr. M.A. Toivio-Kinnucan (College of Veterinary Medicine, Auburn University) and Dr. Michael Miller (Advanced Microscopy and Imaging Laboratory, Auburn University) for excellent electron microscopic support, Dr. Lee Zhang (Genomics and Sequencing Laboratory, Auburn University) for DNA sequencing, Dr. R.C. Bird for discussion of FACS data, and R.R. Young-White for technical expertise in FACS.

References


Immunologia et Therapiae Experimentalis (Warszawa) 50, 379–391.


