IOP Publishing Journal of Physics: Condensed Matter

J. Phys.: Condens. Matter **19** (2007) 395011 (13pp) doi:10.1088/0953-8984/19/39/395011

Recombinant phage probes for *Listeria monocytogenes*

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Received 13 February 2007, in final form 20 March 2007
Published 30 August 2007
Online at stacks.iop.org/JPhysCM/19/395011

Abstract

Monitoring of food and environmental samples for biological threats, such as *Listeria monocytogenes*, requires probes that specifically bind biological agents and ensure their immediate and efficient detection. There is a need for robust and inexpensive affinity probes as an alternative to antibodies. These probes may be recruited from random peptide libraries displayed on filamentous phage. In this study, we selected from two phage peptide libraries phage clones displaying peptides capable of specific and strong binding to the *L. monocytogenes* cell surface. The ability of isolated phage clones to interact specifically with *L. monocytogenes* was demonstrated using enzyme-linked immunosorbent assay (ELISA) and confirmed by co-precipitation assay. We also assessed the sensitivity of phage–bacteria binding by PCR on phage-captured *Listeria* cells, which could be detected at a concentration of 10^4 cells ml^{-1}. In addition, as proof-of-concept, we tested the possibility of immobilizing the affinity-selected phages to a putative biosensor surface. The quality of phage deposition was monitored by ELISA and fluorescent microscopy. Phage–bacterial binding was confirmed by high power optical phase contrast microscopy. Overall, the results of this work validate the concept of affinity-selected recombinant filamentous phages as probes for detecting and monitoring bacterial agents under any conditions that warrant their recognition, including in food products.

1. Introduction

Listeria monocytogenes is a ubiquitous, rapidly growing, Gram-positive bacterium with an unusually broad ecological niche and host range. It causes serious localized and generalized infections in humans and a variety of other vertebrates, including domesticated and wild birds and mammals. In humans, serious, often fatal, disease is most common among pregnant women, newborns and immunocompromised individuals [1]. Several large outbreaks of listeriosis have been associated with contaminated commercial foodstuffs, such as vegetables,

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milk and meat products, on which these bacteria can multiply even at low temperatures [2]. From 1983 onwards, a series of epidemic outbreaks in humans in North America and Europe clearly established listeriosis as an important food-borne infection [3].

Simplified, rapid identification is especially important for pathogenic food-borne bacteria. Among these, usually, the presence of any *Listeria* species in food is an indicator of poor hygiene [4].

Significant efforts have been dedicated to the development of enrichment media and protocols for isolation of *L. monocytogenes* and other *Listeria* species; however, these techniques may take many days to confirm identity. More advanced methods for routine detection, including polymerase chain reaction (PCR), DNA hybridization and enzyme-linked immunosorbent assay (ELISA), offer many potential advantages for the rapid detection of microbial pathogens in environmental samples and food, in comparison with traditional long-term culture methods. Nevertheless, their application for real-time detection and food quality monitoring has significant methodological hurdles.

Advanced bioselective sensors may meet the requests for isolation, concentration of the agents and their immediate detection. The majority of rapid detection biosensors described in the literature have utilized antibodies as bioreceptors. However, while sensitive and selective, antibodies have numerous disadvantages for use as diagnostic biodetectors in food products, including a high cost of production, low availability, limited stability and the need for laborious immobilization methods to sensor substrates [5]. An effective alternative to antibodies are filamentous phages affinity-selected from random peptide libraries for specific, selective binding to biological targets [6]. Filamentous bacteriophages can display on their surface foreign peptides, expressed by foreign DNA introduced in the genome through recombinant modification. In this way, recombinant phage clones may recognize and bind specific targets such as cell surface receptors. Thus, they can act as antibody surrogates, possessing distinct advantages including durability, stability, standardization and low-cost production, while achieving equivalent specificity and sensitivity [7, 5]. In addition, the structure of the outer coat protein of filamentous phage appears to be highly amenable to simple immobilization through physical adsorption directly to the sensor surface, thus providing another engineering advantage while maintaining biological functionality. Numerous phage applications have been proposed [8], including the detection of bacteria [9–11, 6].

In this study, we used a phage display approach to isolate phages/peptides specifically binding to the cell surface of *L. monocytogenes* and analysed some of them in more detail to assess the quality of their bacteria-binding properties.

2. Experimental details

2.1. Bacteria

Listeria monocytogenes ATCC 7644 obtained from the American Type Culture Collection (ATCC, LGC Promochem, Milan, Italy) was propagated in L-Palcam Listeria Selective Enrichment Broth and L-Palcam Listeria Selective Agar (Oxoid). TG1 *Escherichia coli* was used for propagation of phage clones. Stock organisms were maintained in Luria–Bertani (LB) broth containing 20% (v/v) glycerol at $-80\,^{\circ}$C.

2.2. Phage peptide libraries

For the affinity selection procedures, two phage display libraries (pVIII-9aa and pVIII-12aa) were used. These libraries were previously constructed [12, 13] and consist of filamentous

phage displaying random 9- and 12-mer peptides, respectively, fused to the major coat protein (pVIII).

The nonapeptide and dodecapeptide libraries were constructed in the vector pC89 [12], by cloning a random DNA insert between the third and fifth codon of the mature pVIII protein-encoding segments of the pVIII gene. The amino acid (aa) sequences of the amino terminus of the resulting recombinant pVIII are respectively: NH_2-AEGEF.9aa.DPAK . . . in the pVIII-9aa library and NH_2-AEGEF.12aa.GDPAK . . . in the pVIII-12aa library.

2.3. Selection of bacteria-binding phage clones

Library screening was performed using four rounds of affinity selection. In the procedure, complexes of phages with *L. monocytogenes* cells were formed in solution and then separated from unbound phages by centrifugation. Briefly, 10 μl phage library ($\sim 10^{12}$ TU ml^{-1}; TU, ampicillin transducing units) were added to 1 ml *Listeria* cell suspensions, prepared from an overnight culture of *L. monocytogenes* in an orbital shaker–incubator (37 °C, 200 rpm). The overnight culture was washed (three times) by centrifugation (16 000g, 5 min) in sterile phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) and finally resuspended in PBS at OD_{660} 0.5. After incubation of the mixture of *L. monocytogenes* with phages for 1 h at room temperature with gentle agitation on a Rotator Drive STR4 (Pbi, Stuart Scientific, UK), complexes of bacteria with bound phage particles were precipitated by spinning for 5 min at 16 000g, and separated from unbound phage by a series of 10 washing and centrifugation steps (16 000g, 5 min) with 1 ml Tris-buffered saline (TBS)/Tween buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20) each time. Following the final wash, the pellet of cell-bound phages was resuspended in 200 μl of elution buffer (0.1 N HCl, 1 mg ml^{-1} bovine serum albumin (BSA), pH adjusted to 2.2 with glycine) for 20 min at room temperature. The phage eluted from cells was neutralized with 150 μl 1 M Tris–HCl (pH 9.1), and the titre was determined as TU, so as to determine the yield of phage binding to *L. monocytogenes* (i.e. (output phage/input phage)$\times$100), by infecting TG1 cells with the phage, and spreading on ampicillin-containing LB agar plates. The selected phage pool was then amplified by infecting TG1 *E. coli*, superinfection with M13KO7 helper phage ($\sim 10^9$ TU ml^{-1}, Stratagene), 20% polyethylene glycol (PEG) 8000–2.5 M NaCl (PEG/NaCl) double precipitation, and used as input for further rounds of affinity selection against freshly prepared *L. monocytogenes* cells. The number of M13KO7 DNA-containing phage particles was not determined for each sample, as their capsid composition (i.e. recombinant wild-type pVIII) is the same as that of the phagemid-containing particles, which were detected as TU; the typical fusion:helper phage ratio in this kind of preparation is roughly 9:1.

2.4. Screening of positive phage pools

Phage capture ELISA was performed with bacteria by coating the wells with 100 μl of a suspension of *L. monocytogenes* cells (OD_{660} 1.0) in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) and incubating the cells for 1 h at 37 °C, followed by overnight incubation at 4 °C. Wells were washed 10 times with PBS/0.05% Tween-20 on an automatic plate washer, blocked with 5% non-fat dry milk in PBS/0.05% Tween-20 for 1 h at 37 °C with shaking, and washed again.

Phage pools from each round of affinity selection, purified twice by PEG precipitation and resuspended in 100 μl blocking buffer, were added and incubated for 2 h at 37 °C with shaking. Wells were washed again as described above, and an anti-M13 peroxidase conjugate antibody (Amersham Biosciences, Buckinghamshire, UK) was added at a dilution of 1/5000 in blocking

buffer (100 μl/well) for 1 h at 37 °C. After further washing, antibody binding was detected by adding a TMB (3,3′,5,5′-tetramethylbenzidine) liquid substrate system for ELISA, incubated for 45 min at room temperature and stopped with 25 μl of 1 M H_2SO_4. Optical absorbance was recorded at 405 nm (Multiskan Reader, LabSystems). Wild-type vector pC89 served as a negative control for evaluation of background from nonspecific binding.

2.5. Colony immunoscreening

An aliquot of 200 μl of eluted phage suspension from the each ELISA-positive selected pool was used to infect 800 μl of exponentially growing TG1 *E. coli* cells for 45 min at 37 °C. After infection, the bacteria were spread on large (15 cm) LB agar plates containing ampicillin–1% glucose. After overnight incubation at 37 °C, the colonies were recovered from the agar plates with a glass spreader, and 5–10 ml of LB broth containing ampicillin was added until a homogeneous suspension was obtained. 2 ml of LB–ampicillin was inoculated with 10 μl of bacterial suspension to yield an OD_{660} of 0.05–0.1. After growth up to an OD_{660} of 0.4–0.5 at 37 °C, 500 μl of culture was infected with 0.5 μl M13KO7 helper phage ($\sim 10^{12}$ TU ml^{-1}, Stratagene). The infected bacteria were spun down at 6000*g* for 10 min, the pellet was gently resuspended in LB–ampicillin–kanamycin and shaken for 35 min at 37 °C. The infected bacteria were diluted and plated on ampicillin-containing LB plates, and incubated overnight at 37 °C, in order to obtain single colonies. Bacterial colonies, each containing phages from a single library clone, were transferred onto a nitrocellulose (NC) membrane (Millipore) for 1 h at room temperature. The NC membranes were blocked at room temperature for 2 h in a blocking solution (4% BSA in PBS), and then incubated with a suspension of *L. monocytogenes* (OD_{660} 0.5) in PBS/4% BSA for 1 h at room temperature. After washing in PBS/0.05% Tween-20 for 20 min, the membranes were incubated with a 1:1000 dilution of rabbit anti-*Listeria* antibody (Biodesign, Saco, USA) for 1.5 h in PBS/4% BSA. The membranes were washed as described above and then incubated with a 1:15 000 dilution of goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase (AP) (Sigma) for 40 min. The membranes were again washed as described above, and the positive spots on the immunoblots were detected by using the developing AP substrate system (Sigma). Phage particles from bacterial colonies demonstrating the highest signals were characterized by co-precipitation assay, to confirm their specificity for *L. monocytogenes*.

2.6. Co-precipitation assay

In such assay, phage particles interact with *Listeria* cells in suspension, differently from ELISA where they are adsorbed to a solid support, allowing multivalent interactions with bacterial receptors. In this way, the relative binding of the isolated clones to *L. monocytogenes* was estimated with greater sensitivity. Fifty phage clones, from both the 9-mer and 12-mer libraries, identified by colony immunoscreening, were assayed. Each phage clone suspension (500 μl, 10^9 colony-forming units (CFU) ml^{-1} in TBS) was heated at 70 °C for 10 min, mixed with 7.5 μl of 5% Tween-20 and centrifuged at 16 000*g* for 15 min to precipitate phage aggregates, before the addition of 50 μl of a suspension of bacterial cells (OD_{660} 0.5) to the supernatant. The mixture was incubated for 1 h at room temperature (RT) on a Rotator Drive STR4 (Pbi, Stuart Scientific, UK) to allow specific phage–cell binding. Cells were precipitated (16 000*g* for 5 min) and washed five to ten times with 1 ml TBS/0.05% Tween. Negative controls (containing no phage) were prepared by mixing 500 μl TBS with 50 μl aliquots of bacterial suspension. Other negative controls (with no bacteria) containing only 500 μl of phage and 50 μl TBS/0.05% Tween were also prepared. Anti-M13 peroxidase conjugate antibody

(Amersham Biosciences, Buckinghamshire, UK) was added at a dilution of 1/5000 in blocking buffer for 40 min at 37 °C. After three further washing/centrifugation steps, antibody binding was detected by adding TMB substrate and incubating for 30 min at RT; reaction was stopped by adding 1 M H_2SO_4. Each sample supernatant was transferred in a 96-well plate and optical absorbance was recorded at 405 nm (Multiskan Reader, LabSystems). A normalized, mean reactivity and deviation was calculated for three replicates.

The most reactive individual phage clones were amplified and their DNA inserts were sequenced to determine the amino acid sequences of the displayed peptides, as described below.

2.7. PCR and DNA sequencing

Sources of phage DNAs for PCR and sequencing were colonies of infected bacteria. The sequencing primers M13–40 reverse (5′-GTTTTCCCAGTCACGAC-3′) and E24 forward (5′-GCTACCCTCGTTCCGATGCTGTC-3′) were obtained from Proligo, Sigma (Milan, Italy). A sample (1 μl) of the suspended colony was added to the PCR reaction tube, containing 49 μl of mixture for the PCR: 10× Mg free reaction buffer (EuroClone, Milan, Italy) (5 volumes); 50 mM $MgCl_2$ (EuroClone) (5 volumes); Euro-Taq DNA polymerase (5 units μl^{-1} EuroClone) (0.5 volumes); 2.5 mM dNTPs (Roche) (5 volumes); primer M13–40 (10 pmol μl^{-1}) (5 volumes); primer E24 (10 pmol μl^{-1}) (5 volumes); doubly distilled filter-sterilized water (23.5 volumes). The PCR was performed using GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) under the following cycling conditions: one cycle at 94 °C for 5 min; 25 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; and one cycle at 72 °C for 7 min. The PCR products (3 μl) were analysed by agarose gel electrophoresis (1% wt/vol agarose, Sigma, Milan, Italy) in 1× TAE buffer. Gel was stained with ethidium bromide, illuminated on a Dark Reader, and DNA bands were visualized using a Kodak imaging system. The PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced by the DNA sequencing service of CRIBI (University of Padova, Italy) using the M13 primer-40.

2.8. Peptide sequence analysis

The DNA sequences were translated into amino acids by using the 'translate' program on the proteomics server of the Swiss Institute of Bioinformatics Expert Protein Analysis System (ExPASy [http://www.expasy.ch/]). The isoelectric points of the predicted peptide sequences were calculated by using 'compute MW/pI,' also present on the ExPASy proteomics server. The amino acid sequences were aligned according to their similarity by using the CLUSTALX sequence alignment program [14] (available at [http://www.ebi.ac.uk/clustalw/]). GeneDoc (http://www.psc.edu/biomed/genedoc/) was used as a tool for visualizing, editing and analysing multiple sequence alignments of the peptides. Statistical analysis of the insert composition was performed.

2.9. PCR on phage-captured Listeria cells

The functional performance of bacteria-binding phage clones was evaluated with cell suspensions in PBS, ranging from approximately 10^3 to 10^7 cells ml^{-1}, prepared as described above (section 2.3). Bacterial cell counts were confirmed through standard plate count of the cell suspensions.

A 96-well microplate was coated with 100 μl of a suspension of bacteria-binding phage clones ($\sim 10^{10}$ TU ml^{-1}) in carbonate buffer and incubated overnight at 4 °C. Wells were washed 10 times with PBS/0.05% Tween-20 on an automatic plate washer, blocked with 5% non-fat dry milk in PBS/0.05% Tween-20 for 1 h at 37 °C with shaking, and washed again.

Each sample of *L. monocytogenes* was added (100 μl/well) and incubated for 2 h at 37 °C with shaking. Wells were washed again as described above, and phage-captured *Listeria* cells were lysed as follows: 50 μl TE buffer (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA) supplemented with 0.25 μl lysozyme (10 mg ml^{-1}) were added in each well and the mixture was incubated for 15 min at 37 °C with shaking, 0.6 μl proteinase K (20 mg ml^{-1}) was then added and incubated for 15 min at 56 °C. Finally, the plate was incubated at 80 °C for 15 min. One aliquot of each extracted DNA was transferred in a tube, and PCR was performed using primers Lis1B (5′-TTATACGCGACCGAAGCCAAC-3′) and MonoA (5′-CAAACTGCTAACACAGCTACT-3′), leading to a specific product of 660 bp within the listerial *iap* gene [15]. 50 μl of master mix (1.5 mM $MgCl_2$, 1× PCR buffer (EuroClone), a 0.2 mM concentration of each deoxynucleoside triphosphate (Roche), 100 ng of each primer, and Euro-Taq DNA polymerase (1.5 U; EuroClone) was added to each tube; after an initial denaturation step (95 °C, 3 min), 30 PCR cycles (95 °C, 15 s; 58 °C, 30 s; and 72 °C, 45 s) were performed, followed by a final extension step (72 °C, 8 min). The PCR products were analysed on a 1.5% agarose gel containing ethidium bromide (0.2 μg ml^{-1}).

2.10. Binding of phage to quartz

As proof-of-concept, we tested the possibility of immobilizing the affinity-selected phages to quartz (as a putative biosensor surface) by physical adsorption.

Confirmation of phage immobilization on the quartz surface was conducted by ELISA. Small quartz squares were posed on the bottom of a 96-well microplate and incubated for 2 h at 37 °C with 50 μl of phage suspension ($\sim 10^{11}$ virions ml^{-1} in PBS). After incubation, samples were washed ten times with 0.5% Tween-20/PBS to remove unbound phage, and anti-M13 peroxidase conjugate antibody (Amersham Biosciences, Buckinghamshire, UK) was added at a dilution of 1/5000 in 0.4% BSA/PBS (50 μl/well) for 1 h at 37 °C. After further washing, antibody binding was detected by adding 200 μl/well of TMB substrate, incubated for 45 min at room temperature and stopped with 25 μl 1 M H_2SO_4. Optical absorbance was recorded at 405 nm (Multiskan Reader, LabSystems). Quartz with no phage served as a negative control for the evaluation of nonspecific background binding. Results are expressed as mean standard deviation for three replicates of each sample. Differences among samples were established by the Student's t-test analysis by a two population comparison. Statistical significance was considered at a probability $P < 0.05$.

2.11. Binding of Listeria to quartz-immobilized phage

A quartz square (0.5 mm × 0.5 mm) was incubated for 1 h at RT with phage suspension ($\sim 10^{11}$ virions ml^{-1} in PBS), washed ten times with 0.5% Tween-20/PBS to remove unbound phage and soaked in a suspension of *L. monocytogenes* diluted in PBS (OD_{600} 1.0) for 2 h at 37 °C. Subsequently, the square was washed ten times with 0.5% Tween-20/PBS and analysed by phase contrast microscopy (Leica DMRE).

3. Results

3.1. Isolation of recombinant bacteriophages binding to L. monocytogenes

We used two phage displayed random peptide libraries (pVIII-9aa and pVIII-12aa, [12]), containing respectively random nonapeptides and dodecapeptides, to affinity select for peptide binders to cell surface epitopes of *L. monocytogenes*. We performed a selection procedure in which phage clones binding to *Listeria* cells in suspension were separated by centrifugation.

Table 1. Recovery of *L. monocytogenes*-binding phages during selection.

	Round of selection/yield (output/input)			
Phage library	1	2	3	4
pVIII-9aa	1.4×10^{-5}	1.7×10^{-4}	1.1×10^{-2}	2.3×10^{-2}
pVIII-12aa	1.2×10^{-5}	1.7×10^{-4}	1.2×10^{-2}	0.7×10^{-2}

Cell-associated phage pools isolated by acid elution were separately amplified in *E. coli* strain TG1 and used for subsequent rounds of selection. After each round of selection, the yield of phage eluted from the cell surface of *Listeria* was determined prior to amplification. During the selection, the relative portion of *Listeria*-associated phage in amplified phage fractions increased (table 1) indicating effective selection of bacterial binders.

A 1000-fold enrichment for *Listeria*-binding phage occurred during the first three rounds of affinity selection with both libraries. Most of this enrichment was obtained between the second and third rounds of selection (100-fold), with only a minor increase in the first round (10-fold). Yields from third and fourth rounds of selection were similar for both libraries. Phage clone pools from each round of affinity selection were analysed by ELISA.

3.2. Screening of positive phage pools by ELISA

The relative binding of the phage clones from each round of affinity selection to *L. monocytogenes* was estimated by a phage-capture ELISA. In the ELISA assay, phage pools from the selection rounds were added to the wells of a microtitre plate coated with *L. monocytogenes* cells. Following incubation to allow specific binding between cell and phage, non-binding phage particles were washed away and bound phages were revealed using a peroxidase conjugate anti-M13 antibody. Selected clones from the pVIII-9aa library bind to *L. monocytogenes* at a greater degree, in comparison to the pVIII-12aa library. Moreover, phage pools from the third and fourth rounds of affinity selection show, for both libraries, similar reactivity (data not shown).

3.3. Colony immunoscreening

Phage clones from the third and fourth rounds of affinity selection were used to infect TG1 *E. coli* cells. These cells were plated on ampicillin-containing LB plates, and bacterial colonies, each containing a phage population from a single library clone, were screened by a specially developed immunoblot analysis (see section 2). Forty-two phage-containing bacterial colonies, corresponding to the highest signals, were selected and independently propagated for further characterization.

3.4. Co-precipitation assay

To determine specificity of phage binding to *L. monocytogenes* cells, we compared the binding of the selected phage clones with that of phage pC89 vector by a co-precipitation assay (see section 2). In this assay, binding signals are amplified because both phages and bacterial cells are in solution. Thus, phage particles can adopt different conformations allowing monovalent or multivalent interactions with bacterial receptors, which in turn are more available on the cell surface than in cells fixed to the plate.

Table 2. Deduced amino acid sequence of the phage-displayed peptides and their calculated isoelectric point.

Phage clone	ELISA[a]	Amino acid sequence	pI[b]
LI1	1.712	NKKALSPPR	11.17
LI2	2.578	QRKLAAKLT	11.17
LI7	2.237	RKVALPASK	11.17
LN1	2.109	GKKIYMQANFDM	8.50
LN3-9	2.225	MKKVTQVQQVLG	10.00
LN5	1.907	YNKYRAKPQQIM	10.00
LN6	2.710	RKLYALVPPPAP	9.99

[a] ELISA signals are expressed in OD_{405}.
[b] pI, calculated isoelectric point.

Eight clones strongly reacted with *L. monocytogenes* ($OD_{405} > 2$), 16 clones demonstrated a good reactivity (OD_{405} 0.5–2), and the remaining clones showed low ELISA signals ($OD_{405} <$ 0.5). The data on reactivity of representative clones are reported in table 2.

Individual phage clones were characterized by PCR and DNA sequencing and propagated for further analysis.

3.5. DNA sequencing and peptide analysis

The eight most reactive individual phage clones were amplified and their DNA was sequenced to determine the amino acid sequences of the displayed peptide ligands (table 2). Two of the clones turned out to encode the same peptide sequence, as represented by LN3-9 (MKKVTQVQQVLG). They also contained the same genomic sequence, indicating that they correspond to a single phage library clone that was selected twice. The remaining six clones encoded unique peptide sequences. All of the phage-displayed peptides were cationic with calculated isoelectric point values (pI) between 8.50 and 11.17 (table 2).

The phage peptides were aligned and shaded according to their physico-chemical properties (figure 1). Enrichment of specific groups of amino acid residues results from the affinity selection procedure. For example, all of the peptides contain at least two positively charged amino acid residues (R or K, mostly in the amino terminal region) with lysine, always present at position 2 or 3 (figure 1(a)), being the most represented. Moreover, a strong preference for aliphatic amino acid residues can be observed at positions 4 and 5 of the aligned 9-mer peptides (figure 1(b)), a polar residue occupies position 9, and a hydrophobic residue occurs at position 8 (position 7 for LI7 in figure 1(b)). A hydrophobic amino acid residue occupies positions 4, 11 and 12 of the aligned 12-mer peptides, while a polar residue is again found at position 2 (figure 1(c)).

Phage isolates LN6 and LI6 consistently reacted strongly with *L. monocytogenes* in the co-precipitation assay, and thus were used for further analyses aimed at estimating the functional performance of bacteria-binding phage particles.

3.6. PCR on phage-captured Listeria cells

Listeria monocytogenes suspensions were prepared at concentrations ranging from 10^3 to 10^7 cells ml^{-1}. When a capture assay with microplate wells coated with phage LI6 followed by DNA purification was performed, the presence of *L. monocytogenes* was detected by PCR. By using LI6 phage-mediated capture, *L. monocytogenes* could be detected at a concentration of 10^4 cells ml^{-1}, whereas pC89-captured cells (negative control) could be detected only at

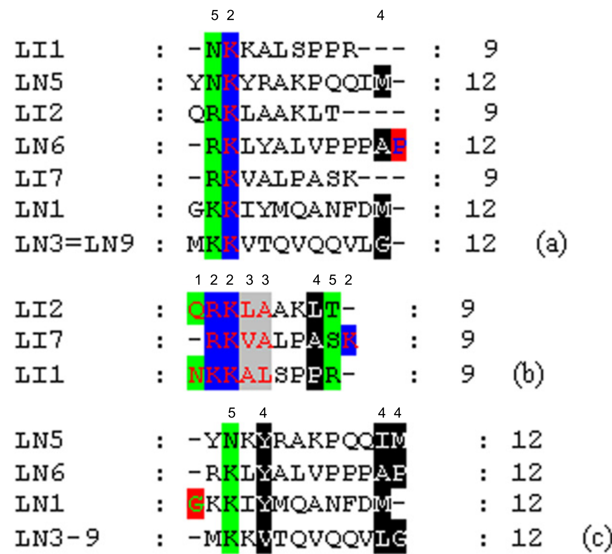

Figure 1. Alignment of phage-displayed peptides. The deduced amino acid sequences from LI1, LI2, LI7, LN1, LN3-9, LN5 and LN6 were aligned by using CLUSTALX (Thompson *et al*, 1994). Dashes indicate gaps used to maximize the alignment. The physico-chemical mode of GeneDoc was used to assign each column of the alignment to one of the 12 predefined groups of physio-chemical properties, indicated by numbers. 1: amphoteric amino acids; 2: positively charged amino acids; 3: aliphatic amino acids; 4: hydrophobic amino acids; 5: polar amino acids.

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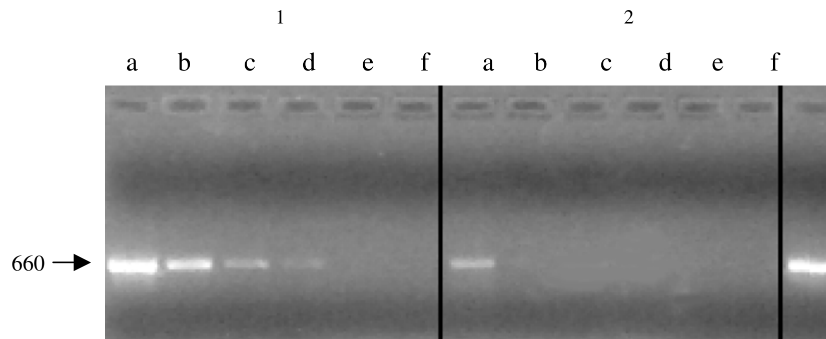

Figure 2. Representative PCR on phage-captured *L. monocytogenes*. Block 1 corresponds to LI6 phage-coated wells and block 2 to pC89 wild-type phage-coated wells. Lanes a–e contain the products of PCR of the respective samples containing 10^7–10^3 cells ml^{-1}; lane f contains the negative control (coated wells and PBS only), and + indicates the position of the positive control (*L. monocytogenes* DNA). The arrow to the left indicates the expected position of the PCR product of 660 bp in length.

the highest concentration (with a PCR product less intense than that of LI6). The results were reproducible with usually at least two of the three parallel reactions being positive (figure 2).

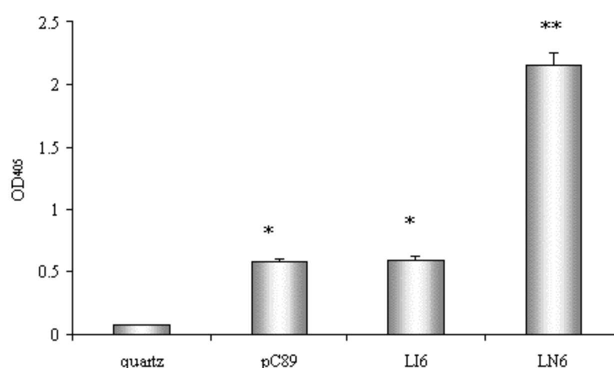

Figure 3. ELISA results of phages immobilization to quartz. ** and * indicate significant difference at $P < 0.01$ and $P < 0.05$ levels, respectively, with reference to the negative control.

3.7. Binding of phage to quartz

Phages LI6 and LN6 were analysed for their ability to be immobilized to quartz, compared to the wild-type pC89. By using ELISA assay, both LI6 and pC89 showed a significant reactivity compared to the negative control, while LN6 appeared to bind even more efficiently (figure 3).

3.8. Binding of Listeria to quartz-immobilized phage

Once phage immobilization to quartz was confirmed, to further assess the quality of bacterial binding we also analysed direct binding of *Listeria* to the immobilized phage by high power optical phase contrast microscopy. In this test, small quartz chips were coated with the selected positive phage by absorption. The chips were soaked in a suspension of *Listeria* cells, washed and analysed microscopically. By this test, we observed in real time the specific binding of bacterial cells to the immobilized phage (figure 4). Confirming ELISA results, LN6 phage covered a higher surface area, as discernible in background, and captured a greater number of *Listeria* cells with respect to LI6. No bacterial cells attached to bare quartz, with the exception of the few adhering in grooves or scratches on the surface.

4. Discussion

Monitoring of food and environmental samples for biological threats, such as *L. monocytogenes*, requires probes that specifically bind biological agents and ensure their separation, purification and detection. Isolation, concentration of the agents and their immediate detection may be performed using advanced bioselective absorbents and biosensors. The key elements of biosorbents and biosensors are affinity probes that recognize specific structural features on the surface of the threat agent. The probes (usually antibodies) are immobilized on the solid matrices of biosorbents and biosensors, allowing capturing of the agents and their registration [11].

However, broad application of antibodies is limited because of their high production and operational costs and a low resistance to unfavourable environmental conditions. Therefore, there is a need for alternative robust and inexpensive affinity probes that would capture defined groups of biological threats in highly diverse environmental conditions and allow their immediate and efficient detection.

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

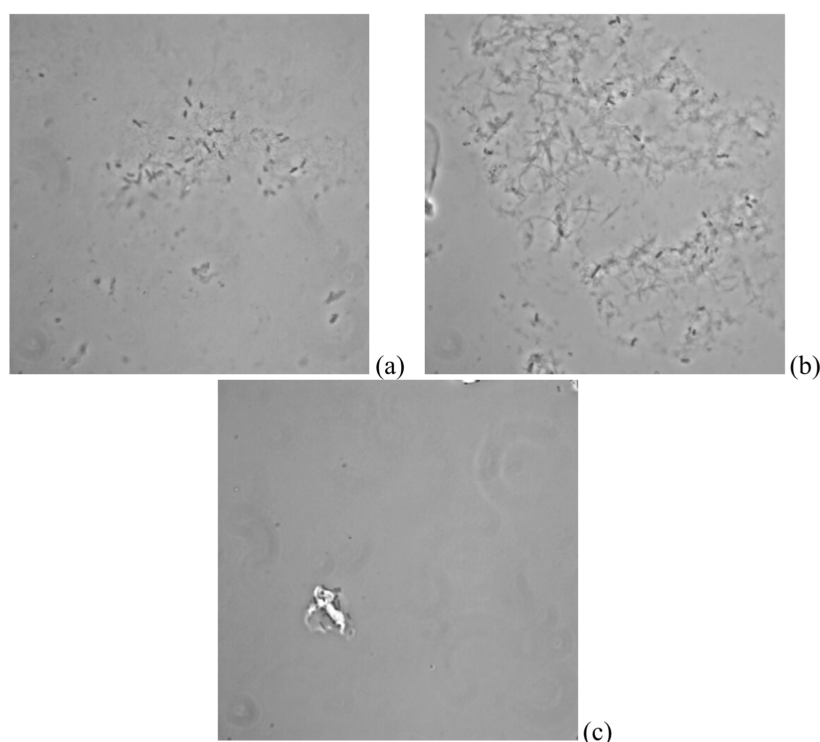

Figure 4. Phase contrast microscopy images of *L. monocytogenes* attached to the surface of quartz previously adsorbed with the affinity-selected phages LI6 (a) and LN6 (b) (discernible in background). Quartz with no phage is shown as a negative control. (c) 1000× magnification.

random peptide libraries displayed on filamentous phage. The phage-derived probes, burdened with 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-assembly of virions or their composites on solid surfaces.

Phage display technique is a new approach for development of phage-derived diagnostic probes [11, 5, 8]. Landscape phages have been shown to serve as substitutes for antibodies against various soluble and cell-displayed antigens and receptors [16–18] including bacterial cells and spores [6, 11]. The phage probes have been used in ELISA and thickness shear mode quartz sensors to detect bacterial and mammalian antigens [17, 5].

In this study, we used the two phage displayed random peptide libraries pVIII-9aa and pVIII-12aa, which contain random nonapeptides and dodecapeptides, respectively, fused to 150–300 copies of the major coat protein pVIII [12], to isolate phage clones displaying peptides capable of specific and strong binding to whole *L. monocytogenes* cell surface.

The above phage libraries resulted to contain several potential probes for surface markers of *L. monocytogenes*, which could be isolated by multistage selection using whole bacteria as a selector. We elaborated a selection procedure where complexes of phages with bacterial cells were formed in solution and were separated from unbound phages by precipitation, resulting in the discovery of four 9-mer and four 12-mer *Listeria*-binding peptides. The phage clone LN3-9, encoding the peptide sequence MKKVTQVQQVLG, was selected twice, whereas the remaining seven peptide sequences (table 2) correspond to single isolates. A conventional amino acid alignment could not reveal any obvious consensus pattern among the groups of

peptides; however, it was evident that strong selection pressure had occurred for amino acid residues with similar physico-chemical properties (figure 1).

The ability of isolated phage clones, possessing specific peptide sequences, to interact specifically with *L. monocytogenes*, the selector bacterium, was demonstrated using ELISA and confirmed by co-precipitation assay. We also assessed sensitivity of phage–bacteria binding by PCR on phage-captured *Listeria* cells, which could be detected at a concentration of 10^4 cells ml^{-1}.

In addition, as proof-of-concept, we tested the possibility of immobilizing the affinity-selected phages to quartz (as a putative biosensor surface) by physical adsorption. Confirmation of the immobilization of the two most reactive phage clones on the quartz surface was obtained by ELISA. Moreover, visual observation by high power optical phase contrast microscopy confirmed attachment of *Listeria* cells to the surface of quartz coated with filamentous phages.

Affinity-selected landscape phage probes for *Salmonella typhimurium* with the specificity, selectivity and affinity of monoclonal antibodies were demonstrated [6] and utilized as probes for biodetectors [10]. Landscape phages discovered in this work may find application as biosorbents and diagnostic probes for monitoring of *L. monocytogenes* by various devices in which antibodies have been used, with the previously mentioned advantages over antibodies. For example, they may be used for separation and purification of bacteria prior to their identification with PCR, immunoassays, flow cytometry or other methods. Furthermore, they may find application as biorecognition elements of real-time biosensor devices, as they specifically and exclusively bind target cells.

5. Conclusion

Along with other recently published data [6], the results of this work validate the concept of affinity-selected recombinant filamentous phages as probes for separation, concentration and detection of biological threat agents.

Sensors prepared with phage as probes could be an effective analytical method for detecting and monitoring quantitative changes of bacterial agents under any conditions that warrant their recognition, including in food products. We believe that this new generation of robust and inexpensive phage-derived probes will serve as efficient substitutes for antibodies in isolation, concentration and detection systems employed for food and environmental monitoring.

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