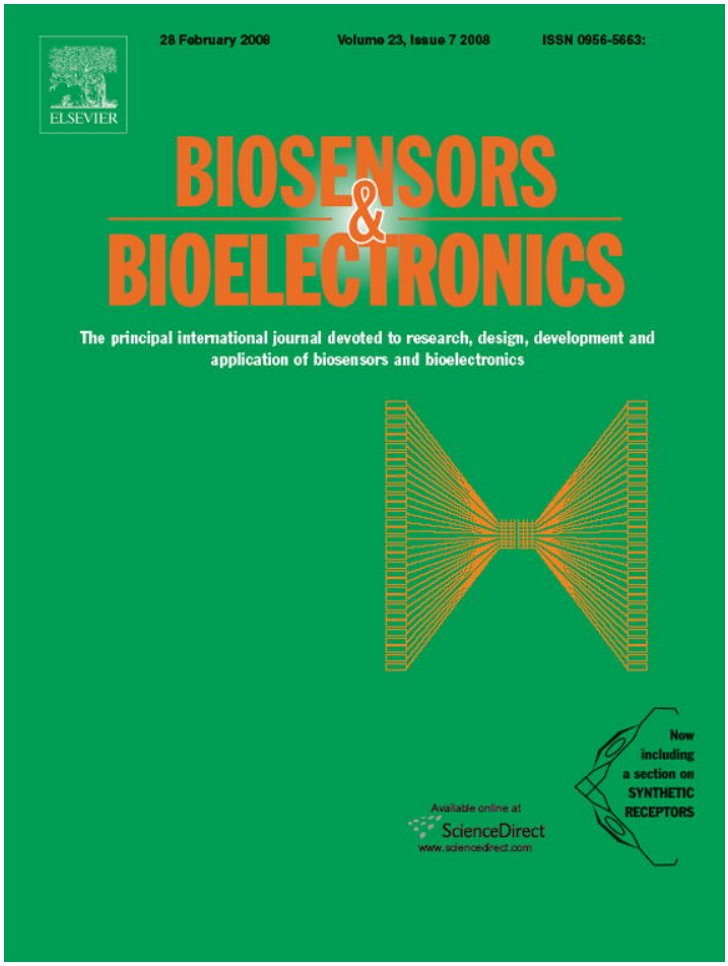

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Biosensors and Bioelectronics 23 (2008) 1137–1144
BIOSENSORS & BIOELECTRONICS
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Specific and selective probes for *Pseudomonas aeruginosa* from phage-displayed random peptide libraries

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Received 18 August 2007; received in revised form 1 November 2007; accepted 2 November 2007
Available online 9 November 2007

Abstract

The design of novel biosensors for the detection of biological threats, such as *Pseudomonas aeruginosa*, requires probes that specifically bind biological agents and insure their immediate and efficient recognition. Advanced bio-selective sensors may meet the requests for isolation, concentration of the agents and their real-time detection. There is a need for robust and inexpensive affinity probes alternative to antibodies. These probes may be recruited from random peptide libraries displayed on filamentous phage. In this study, we identified from two phage-displayed random peptide libraries phage clones displaying peptides capable of specific and strong binding to *P. aeruginosa* cell surface. The ability of the phage clones to interact specifically with *P. aeruginosa* was demonstrated by using enzyme-linked immunosorbent assay (ELISA). We assessed selectivity of phage–bacteria-binding by comparing the binding ability of the selected clones to the selector bacterium and a panel of other bacterial species; we also demonstrated by dot spot and immunoblotting that the most reactive and selective phage peptide bound with high avidity the bacterial cell surface. In addition, as proof-of-concept, we tested the possibility to immobilize the affinity-selected phage to a putative biosensor surface. The quality of phage deposition was monitored by ELISA, and 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 clinical-based diagnostics and possibly biological warfare applications.

Keywords: Phage-display; Biosensor selective probes; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that is among the most common agents of nosocomial infections (Campa et al., 1993; Sadikot et al., 2005) and the third most common bacterial isolate from blood borne infections (Osmon et al., 2004). In particular, it is commonly associated with progressive, chronic respiratory infection in patients with cystic fibrosis (CF) and other causes of airway derangement (Davis et al., 1996). When conditions are favourable, *P. aeruginosa* can infect wounds, burnt areas and the urinary and respiratory tracts, and it may also be involved in bronchiectasis (Ho et al., 1998), endocarditis (Komshian et al., 1990), meningitis and various other pathological conditions (van Delden and Iglewski, 1998). It can be extremely versatile from a nutritional standpoint, in fact it can grow at the expense of a large variety of organic substrates or in very simple nutritional environments without any organic growth factor (Spiers et al., 2000). The widespread habitat of *P. aeruginosa* in nature, which includes soil, water, food and the surfaces of plants and animals, makes very difficult controlling the organism in hospital settings, and prevention of contamination is almost impossible. The list of materials from which this bacterium can be isolated is almost endless and, despite the recent development of different detection methods with no need for target amplification and enrichment, no sufficiently quick and reliable diagnostic systems for *P. aeruginosa* detection are available to date.

Advanced bio-selective sensors may meet the requests for isolation, concentration of the agents and their immediate real-time detection. The majority of rapid detection biosensors described in the literature has utilized antibodies as bioreceptors; however, while sensitive and selective, antibodies have

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doi:10.1016/j.bios.2007.11.001

numerous disadvantages for use as diagnostic biodetectors in clinical medicine, including high cost of production, low availability, great susceptibility to environmental conditions (Shone et al., 1985) and the need for laborious immobilization methods to sensor substrates (Petrenko and Vodyanoy, 2003). An effective alternative to antibodies may be short peptides affinity-selected from random phage-displayed peptide libraries for specific, selective binding to biological targets (Sorokulova et al., 2005). Filamentous bacteriophages can display on their surface foreign peptides, expressed by DNA sequences introduced in the genome through recombinant modification, able to recognize and bind specific targets, such as cell surface receptors. Thus, phage-displayed peptides can act as antibody surrogates, possessing distinct advantages including durability, standardization and low-cost production, possibility to be produced also for targets too small or toxic for antibody arising, while achieving equivalent specificity and sensitivity (Petrenko et al., 1996; Petrenko and Vodyanoy, 2003). In addition, the short protein structure appears to be highly amenable to simple immobilization through physical adsorption and to more correct orientation onto the sensor surface, thus providing another engineering advantage while maintaining biological functionality (Olsen et al., 2006; Carnazza et al., 2007).

Phage-display has become routinely used for the isolation of peptide ligands to a wide variety of targets, including small molecules (Saggio and Laufer, 1993), receptors (Balass et al., 1993) and whole-cell epitopes (Sorokulova et al., 2005; Carnazza et al., 2007; Cwirla et al., 1990; Yu and Smith, 1996). In particular, this technology represents a powerful tool for the selection of peptides binding to specific motifs on whole cells since it is a non-targeted strategy, which also enables the identification of surface structures that may not have been considered as targets or have not yet been identified (Bishop-Hurley et al., 2005).

In this study, we used a whole-cell phage-display approach to isolate peptides specifically binding to the cell surface of *P. aeruginosa*. The most specific and selective phage peptide was cationic and strongly bound to the bacterial cell surface, as demonstrated by dot spot and Western immunoblot analyses. This phage clone was immobilized onto quartz and its ability to bind and capture *Pseudomonas* cells was investigated.

2. Materials and methods

2.1. Bacteria

P. aeruginosa ATCC 27853 obtained from the American type culture collection (ATCC, LGC Promochem, Milan, Italy) was propagated in Luria Bertani (LB) broth and Pseudomonas Isolation Agar (Oxoid). TG1 *Escherichia coli* was used for propagation of phage clones. *Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 7644, *Shigella flexneri* ATCC 12022, *E. coli* LE392 (F^- strain) and *Bacillus subtilis* ATCC 6633 were used in selectivity assessment of phage–bacteria-binding. Stock organisms were maintained in LB broth (or TSB, tryptone soya broth, for *Listeria* and *Bacillus*) containing 20% (v/v) glycerol at −80 °C.

2.2. Bacteriophage

Filamentous phage as specific, selective probes for *P. aeruginosa* were derived from two phage peptide libraries (pVIII-9aa and pVIII-12aa) through previously described affinity-selection procedures (Carnazza et al., 2007). These libraries consist of filamentous phage particles displaying random 9 and 12mer peptides, respectively, fused to the major coat protein (pVIII). The nonapeptide and dodecapeptide libraries were constructed in the vector pC89 (Felici et al., 1991), by cloning a random DNA insert between the third and fifth codon of the mature pVIII-encoding segments of gene VIII (Luzzago and Felici, 1998).

2.3. Phage peptide selection

Library screening was performed using six rounds of affinity selection. Selection against *P. aeruginosa* whole cells was performed by incubating 10^{12} phages with *P. aeruginosa* cells (OD_{660} 0.5) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4; 1 ml) for 60 min at room temperature with gentle agitation. Bacteria and phages were precipitated by spinning for 5 min at 16,000 × *g*, and separated from unbound phage in solution by a series of 10 washing and centrifugation steps (16,000 × *g*, 5 min) with 1 ml TBS/Tween buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20) each time. Bound phages were pelleted with cells and finally eluted with 200 μl of 0.2 M glycine–HCl (pH 2.2) with gentle shaking at room temperature for 20 min, followed by neutralization with 150 μl of 1 M Tris–HCl (pH 9.1).

Phage eluted from the sixth and final round of affinity selection against *P. aeruginosa* was used to infect TG1 *E. coli* cells. These cells were plated on ampicillin-containing LB agar plates to select for bacteria containing phage. Bacterial colonies, each containing phages from a single library clone, were randomly selected and propagated for subsequent analyses.

2.4. Phage-capture ELISA

Wells of a 96-well ELISA dish were coated overnight at 4 °C with 100 μl suspensions of *P. aeruginosa* (OD_{660} 2.0) in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6); 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; washed again; reacted with phage clones (100 μl in blocking buffer for 2 h at 37 °C with shaking); washed again; reacted with 100 μl of anti-M13 peroxidase conjugate antibody (Amersham Biosciences, Buckinghamshire, UK) at a dilution of 1/5,000 in blocking buffer for 1 h at 37 °C; washed again; reacted with 200 μl of TMB (3,3′,5,5′-tetramethylbenzidine) liquid substrate system for ELISA for 45 min at room temperature and stopped with 25 μl of 1 M H_2SO_4. Wells were then read on a kinetic plate reader at 405 nm (Multiskan Reader, LabSystem). Phage particles bearing no recombinant insert, from superinfection of phagemid vector pC89-containing cells, served as a negative control for evaluation of background from non-specific binding.

2.5. DNA sequencing and peptide analysis

Sources of phage DNA 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 the 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 U/μl EuroClone) (0.5 volumes); 2.5 mM dNTPs (Roche) (5 volumes); primer M13-40 (10 pmol/μl) (5 volumes); primer E24 (10 pmol/μl) (5 volumes); doubly distilled filter-sterilized water (23.5 volumes). The PCR was performed using GeneAmp PCR System 2400 (PerkinElmer, 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; one cycle at 72 °C for 7 min. PCR products (3 μl) were analyzed by agarose gel electrophoresis (1%, w/v agarose, Sigma, Milan, Italy) in 1× TAE buffer. Gel was stained with ethidium bromide, illuminated on Dark Reader, and DNA bands were visualized using a Kodak imaging system. PCR products were purified using QIAquick PCR purification Kit (QIAGEN) and sequenced by DNA sequencing service of C.R.I.B.I. (University of Padova, Italy) using the M13 primer -40.

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.

2.6. Membrane preparation

Outer membrane proteins (OMPs) were purified from *P. aeruginosa* ATCC 27853, modifying what described by Bonfiglio and Livermore (1991). Briefly, *P. aeruginosa* cells were resuspended in 30 mM Tris–HCl (pH 8.0)/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (PMSF) buffer and disrupted by 3 s × 60 s bursts of sonication at 70% pulse, with ice bath cooling, using a model HD 2070 MS 72 Sonicator (Bandelin Sonopuls, Berlin, Germany). The sonicates were centrifuged at 8000 × *g* for 20 min, and the clear cell-free supernatant ultracentrifuged at 100,000 × *g* for 1 h at 4 °C in a Ti70 rotor (Beckman L80 Optima) for separation of membrane fractions. Pellets were washed twice with 30 mM Tris–HCl (pH 8.0) buffer and resuspended in 600 μl 20% (w/v) sucrose in the same buffer. This sample (300 μl each) was layered on top of a two-step (60–70% (w/v) sucrose in 30 mM Tris–HCl buffer or a three-step gradient (30–50–70% (w/v) sucrose in 30 mM Tris–HCl buffer). The gradients were ultracentrifuged at 100,000 × *g* in a swing-out rotor SW 55 Ti (Beckman L80 Optima) for 15 h at 4 °C, thereby separating the outer and inner membranes, which formed distinct bands. The lower band, on the 70% sucrose layer, containing the outer membrane, was recovered, washed with 30 mM Tris–HCl (pH 8.0) buffer and then ultracentrifuged at 100,000 × *g* in a 70Ti rotor (Beckman L80 Optima) for 1 h at 4 °C. The resulting pellet was resuspended in 400 μl of Millipore water and stored at −20 °C.

2.7. Dot spot

For dot spot assays (modified from Dai et al., 2003), 50 μl extracted *P. aeruginosa* membranes were spotted onto nitrocellulose membrane disks (PROTRAN 0.45 μm, Schleicher and Schuell), then blocked with 4% BSA (powder in PBS) for 30 min, at room temperature with gentle agitation, washed five times with PBS/0.05% Tween 20/4% BSA and air-dried for about 30 min. 50 μl/disk of the phage clones (the positive recombinant phage P9b or the wild-type vector pC89) were incubated for 2.5 h at 37 °C. Unbound phage were removed by five washing steps with PBS/0.05% Tween 20/4% BSA, and nitrocellulose disks were incubated for 1 h at 37 °C with anti-M13 peroxidase conjugate antibody diluted 1/5,000 in blocking buffer. After washing, reaction was assayed with 50 μl/disk of 3,3′-diaminobenzidine (DAB, Sigma, Milan, Italy) by colour development. Negative controls (containing no membranes) were prepared by spotting 50 μl TBS onto nitrocellulose membrane disks. As well, other negative controls (for assessing binding selectivity for cell receptors) were prepared by spotting 50 μl extracted *E. coli* LE392 (F^- strain) membranes.

2.8. Immunoblotting

For Western immunoblot analyses, OMPs from *P. aeruginosa* were boiled for 5 min in sodium dodecyl sulfate (SDS) reducing buffer prior to being separated at 150 V on Bio-Rad SDS-12.5% polyacrylamide gel electrophoresis (PAGE) minigels (Laemmli, 1970).

Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (0.45 mm, Schleicher and Schuell) by using the Mini-Trans-Blot transfer cell (Bio-Rad) for 12–16 h at 23 V. The membranes were blocked at room temperature for 1 h in blocking buffer (4% bovine serum albumin in PBS), washed for 30 min with PBS/0.05% Tween 20/4% BSA and then incubated with 10^{11} virions of phage-displayed peptides for 2 h at 37 °C in a BIO-RAD MINI Protean II MULTISCREEN system.

After being washed in PBS/0.05% Tween 20/4% BSA/4 M urea for 20 min (urea was added in washing buffer in order to assess the binding avidity of phage), the membranes were incubated with a 1:5,000 dilution of anti-M13 peroxidase conjugate antibody in blocking buffer for 1 h. After the membranes were washed as described above, the bands on the immunoblots were detected by using DAB (Sigma, Milan, Italy) for 15–20 min, dried and preserved in plastic envelop. SDS-PAGE broad-range prestained markers (Bio-Rad) were used to estimate the size of the bands binding the recombinant phage.

2.9. Binding of phage to quartz

As proof-of-concept, we tested the possibility to immobilize the affinity-selected phage to quartz (as a putative biosensor surface) by physical adsorption. Phage immobilization on the quartz

surface was confirmed 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 (~10^{11} virions/ml in PBS). After incubation, samples were washed 10 times with PBS/0.5% Tween 20 to remove unbound phage, and anti-M13 peroxidase conjugate antibody was added at a dilution of 1/5,000 in PBS/0.4% BSA (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, LabSystem). Quartz with no phage served as a negative control for evaluation of nonspecific background binding. Results are expressed as mean-standard deviation for three replicates of each sample. Differences among samples were established by Student's *t*-test analysis by a two-population comparison. Statistical significance was considered at a probability $P < 0.05$.

2.10. Binding of Pseudomonas to quartz-immobilized phage

20 μl phage suspension (~10^{11} virions/ml in PBS) was spotted onto a quartz square (0.5 cm × 0.5 cm), incubated for 1 h at 37 °C, and washed 10 times with PBS/0.5% Tween 20 to remove unbound phage. A negative control, consisting of a bare, clean quartz square was used for comparison. An overnight culture of *P. aeruginosa* was washed by centrifugation with PBS and diluted to prepare a final 6 ml suspension in PBS of OD_{600} 0.5. The quartz samples were soaked in the bacterial suspension for 2 h at 37 °C and observed by phase contrast microscopy (Leica DMRE). Sequential digital images of cell binding were acquired using a CCD camera (Leica DC300F) in 20 min intervals for up 2 h. Following cell deposition for a specified duration, the quartz samples were removed using sterile tweezers, rinsed of excess cells by immersion in PBS/0.5% Tween 20 (3×), and examined under phase contrast oil immersion (1000×). A quantitative evaluation of cell binding was performed using the Scion Image Software (Windows version of NIH Image Software), in terms of integrated density (I.D. = $N \times (M - B)$, where N is the number of pixels in the selection, M is the average grey value of the pixels, and B is the most common pixel value) (Carnazza et al., 2006). Results are expressed as mean-standard deviation for three replicates of each sample. Differences among samples were established by Student's *t*-test analysis by a two-population comparison. Statistical significance was considered at a probability $P < 0.05$.

3. Results

3.1. Isolation of phage-displayed peptides binding to P. aeruginosa

We used two random peptide phage-display libraries (pVIII-9aa and pVIII-12aa), containing, respectively, random nonapeptides and dodecapeptides, to affinity select for peptides that bound to cell surface epitopes of *P. aeruginosa* ATCC 27853 strain. We used a selection procedure in which phage clones binding to *Pseudomonas* cells in suspension were separated by centrifugation. Cell-associated phages 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 *Pseudomonas* was determined prior amplification. During the selection, the relative portion of *Pseudomonas*-associated phage in amplified phage fractions increased from one round to another indicating effective selection of bacterial binders. Phage clone pools from each round of affinity selection were analyzed by ELISA, showing significant reactivity ($OD_{405} > 2$) since the second round (data not shown). Following the sixth round, individual phage clones were randomly selected from the eluted phage population and independently propagated for further screening.

3.2. Specificity and selectivity of phage binding to P. aeruginosa

To determine specificity, as the ability of a phage displaying a specific peptide to interact with a target bacterium, we compared the binding of the selected phage clones with that of phage pC89 vector in a phage-capture ELISA assay. Selected clones from the pVIII-9aa library bound to *P. aeruginosa* at a greater degree, in comparison to the pVIII-12aa library. Representative clones from each library demonstrating the highest ELISA signals were investigated for their ability to preferentially interact with the select target in comparison to other potential targets. In particular, their binding was compared to *P. aeruginosa* and control strains of another Gram-negative, *S. typhimurium*, and of a Gram-positive, *L. monocytogenes*. In ELISA assay, each organism was incubated with an equal amount of each phage, and bound virions were detected by using anti-M13 peroxidase conjugate antibody and then TMB substrate colour development.

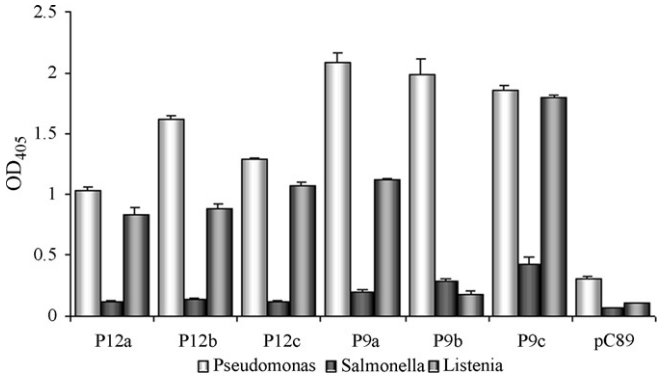

Fig. 1. ELISA results of bacteria-binding by representative selected phage clones (P12, phage clones selected from pVIII-12aa library; P9, phage clones selected from pVIII-9aa library). Mean OD_{405} is the average of three separate experiments. Error bars indicate standard deviations. Paired *t*-test indicates an extremely significant reactivity ($P < 0.0001$) of clones from both libraries for *Pseudomonas aeruginosa* in comparison to the control (pC89), and of each clone for binding to *P. aeruginosa* with reference to the other challenge bacteria, except for P9c that binds to *P. aeruginosa* as well as to *Listeria monocytogenes* (not significant difference). P9 clones bind very significantly ($P < 0.001$) to *S. typhimurium*, whereas difference is not significant for P12 clones, compared with pC89. Binding of both P12 and P9 clones to *L. monocytogenes* is statistically extremely significant ($P < 0.0001$) in comparison to the control (pC89), except for P9b that shows not significant reactivity.

No phage clone significantly bound to *S. typhimurium*, whereas clones from both libraries reacted also with *L. monocytogenes*, except for P9b (Fig. 1).

The selectivity of P9b phage binding to *P. aeruginosa* was furthermore assessed in comparison with other Gram-negative bacteria, still belonging to Enterobacteriaceae (*S. flexneri* and *E. coli*), and another Gram-positive strain (*B. subtilis*). ELISA assay demonstrated 10 times greater binding of phage to *P. aeruginosa* versus all the challenge bacteria (Fig. 2), confirming P9b to be the most specific and selective phage clone for *P. aeruginosa*.

DNA was isolated from the clone and the peptide-encoding region sequenced to determine the amino acid sequence of the displayed nonapeptide ligand. Phage clone P9b displayed the foreign peptide QRKLAAKLT, which resulted cationic with a calculated isoelectric point value (p*I*) of 11.17.

3.3. Characterization of the binding specificity of the phage-displayed peptide

We tested the P9b phage clone for its ability to bind to OMPs from *P. aeruginosa* ATCC 27853 strain. Dot spot assay showed that outer membrane fraction of *P. aeruginosa*, separated by both two-step and three-step gradient ultracentrifugation, was recognized and specifically bound by P9b phage clone, whereas no significant reaction was detected for P9b with membrane extracted with the same method from *E. coli* F^- strain either for the control pC89 with both bacteria OMPs (Fig. 3).

The Western immunoblot analysis revealed that P9b phage clone bound with high avidity to a band with a mobility of ca. 42 kDa (Fig. 4), confirming its selective and specific binding to *P. aeruginosa* surface.

3.4. Binding of phage to quartz

P9b phage was analyzed for its ability to be immobilized to quartz, by using ELISA assay. It showed a significant reactivity compared to the negative control ($P < 0.05$).

3.5. Binding of Pseudomonas to the quartz-immobilized phage

Once confirmed P9b phage immobilization to quartz, binding of *P. aeruginosa* cells to the immobilized phage was observed

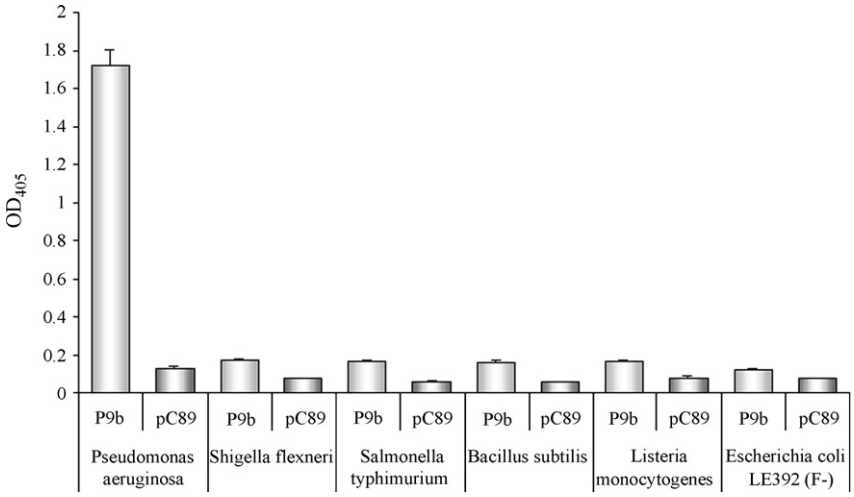

Fig. 2. Selectivity of phage P9b as determined by ELISA. Mean OD_{405} is the average of three separate experiments. Error bars indicate standard deviations. Paired *t*-test indicates an extremely significant difference ($P < 0.0001$) for P9b binding to *P. aeruginosa* with reference to both the control (pC89) and all the challenge bacteria.

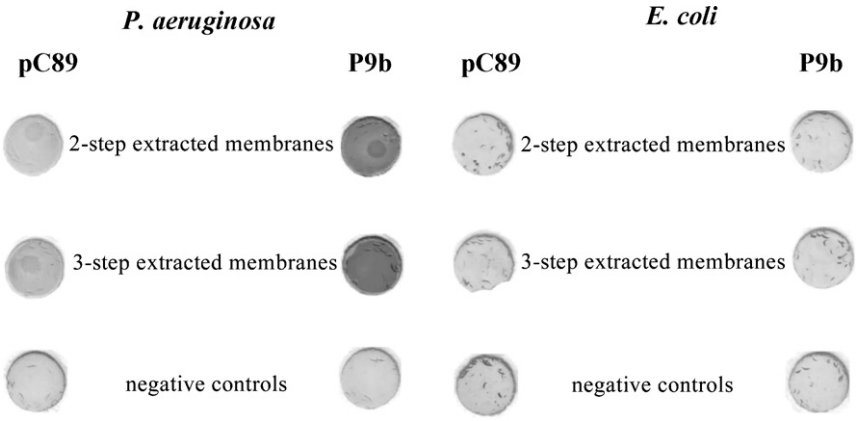

Fig. 3. Dot spot assay with the selected phage-displayed peptide (see Section 2.7).

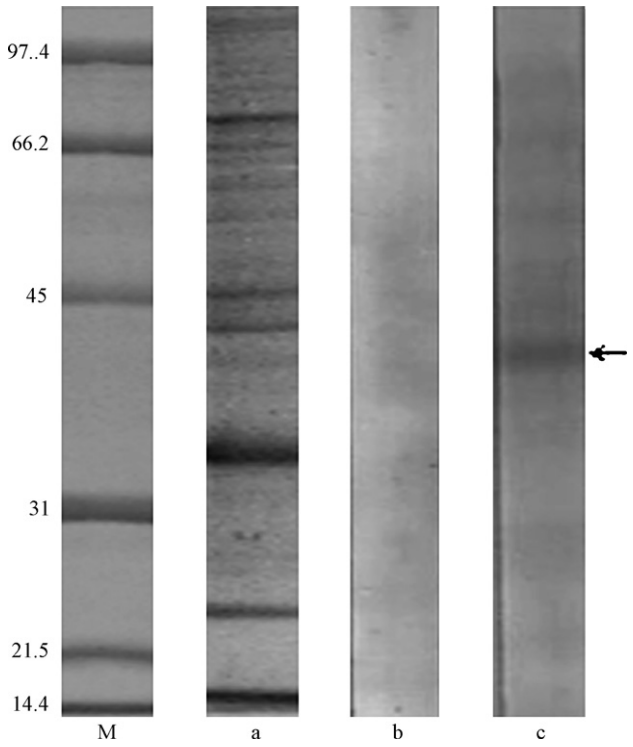

Fig. 4. Immunoblot analysis with the selected phage-displayed peptide. OMPs isolated from *P. aeruginosa* ATCC 27853 strain were separated by SDS-PAGE (a) and blotted onto nitrocellulose membranes (b–c). The membranes were probed separately with pC89 vector (b) and the phage clone P9b (c), respectively. SDS-PAGE broad-range pre-stained markers were used (M, Mw indicated in kDa).

directly by high-power optical phase contrast microscopy. In this test, we observed in real-time specific binding of bacterial cells to the immobilized P9b phage. A poor adhesion was observed for the reference quartz surface, mainly in correspondence of grooves or scratches (Fig. 5A), while a significantly increasing number of cells was captured by the immobilized phage, showing very crowded cell aggregations after 2 h of deposition (Fig. 5B). The surface coverage, in terms of I.D. mean values measured at 2 h, for *P. aeruginosa* on the P9b-coated surface was extremely greater ($P < 0.0001$) than on the bare quartz.

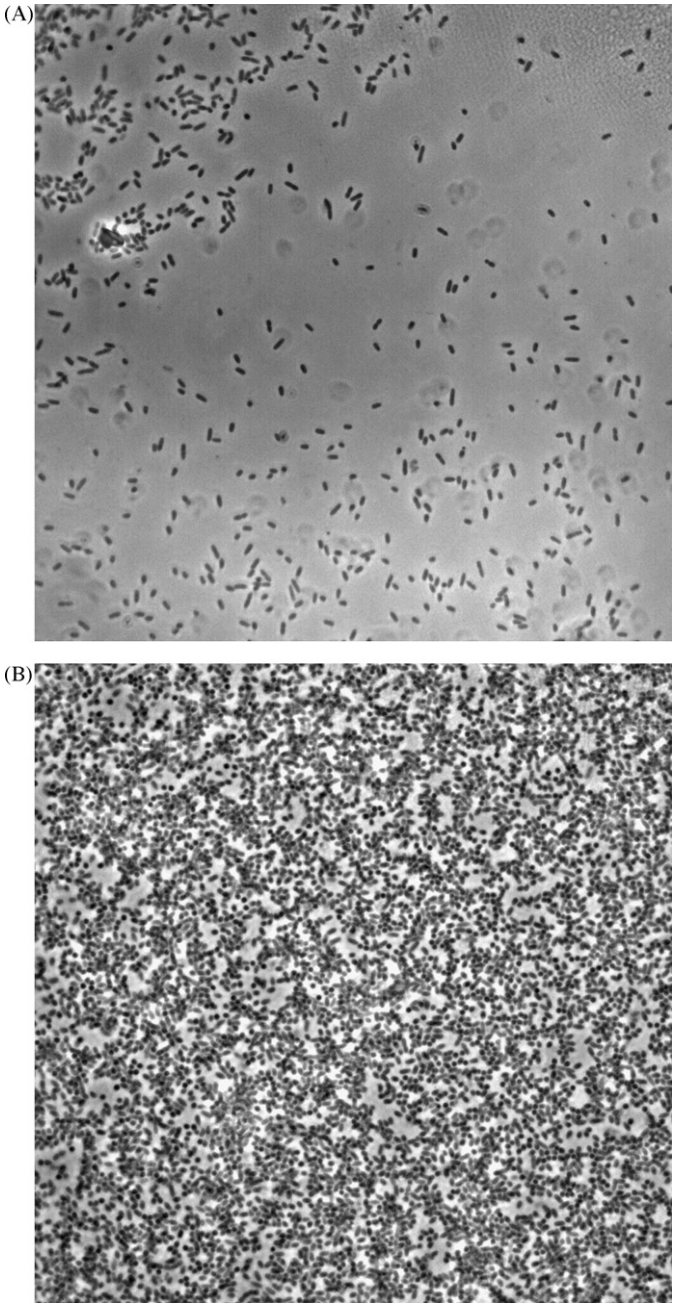

Fig. 5. Phase contrast microscopy images of *P. aeruginosa* attached to the surface of quartz with no phage as negative control (A), and to quartz previously adsorbed with the affinity-selected P9b phage (B). 1000× magnification.

4. Discussion

When attempting to design a new biosensor a question is what parameter the sensor must detect and what kind of probe is necessary to be used. The essential element of any detector device is the probe that specifically binds the target and, as a part of the analytical platform, generates a measurable signal. The majority of sensor devices utilize many polymers with definite roles, either in the sensing mechanism or through immobilizing the species responsible for sensing of the analyte component (Adhikari and Majumdar, 2004; Guilbault and Jordan, 1988). This has become possible only when polymers may be tailored for particular properties: are easily processed and may be selected to be inert in the environment containing the analyte. But it is not so easy to take out the right probe, in particular for biological components and, what is more important, in an in vivo system.

This research demonstrates, along with other our previously published data (Carnazza et al., 2007), that phage-displayed peptides show promise as probes for biosensor applications. In fact, in optimized form, this phage probe could be used to build a micro-biosensor system in which biological sensing element is the selected phage-displayed peptide.

In this work, specificity of isolated phage clones for *P. aeruginosa* was demonstrated using phage-capture ELISA, and the binding of representative clones to *P. aeruginosa* was compared to a panel of select bacteria, revealing high selectivity of

the phage P9b (QRKLAAKLT) to the selector bacterial strain (Fig. 2). Dot spot assay demonstrated that P9b specifically bound *Pseudomonas* cell outer membrane (Fig. 3), recognizing probably a surface receptor corresponding to the denatured epitope of ca. 42 kDa indicated by Western blot analysis (Fig. 4). This band did not match any known either proposed major outer membrane protein of *P. aeruginosa* (http://www.cm.ubc.ca/bobh/omps/); perhaps one of the characterized mucin-binding adhesins could be involved in the interaction (Carnoy et al., 1994). In any case, these data suggest that P9b phage particles strongly bind to a specific motif on the bacterial cell surface.

Numerous highly specialized, variable major and minor proteins, interlaced among the LPS, may be 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, but may vary in primary structure. 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. In addition, no significant reaction was detected for P9b clone with membrane extracted from *E. coli* F^- strain in dot spot assay. Therefore, it is reasonable to hypothesize that the phage-targeted receptor on the surface of *P. aeruginosa* differs from *E. coli* receptors, as confirmed also by the absence of cross-reactivity of the specific and highly selective phage clone selected for *P. aeruginosa* versus Enterobacteria in general and particularly *E. coli*.

The obtained data strongly suggest that the peptide selected by phage-display and characterized in this work may find application as biosorbent and diagnostic probe for monitoring *P. aeruginosa* by various devices in which antibodies have been used to date. For example, it may be used for separation and purification of bacteria prior to their identification with polymerase chain reaction, immunoassays, flow cytometry, or other methods. Furthermore, it may find application as bio-recognition element of real-time biosensor devices, since it does not cross-react with both Enterobacteria and some Gram-positive bacterial species and selectively captures *P. aeruginosa* cells when adsorbed onto a putative biosensor surface as quartz. We believe that this new generation of selective and inexpensive phage-derived probes will serve as efficient substitutes for antibodies in separation, concentration and detection systems employed for clinical and environmental monitoring.

Recombinant peptides selected by phage-display selectively recognize and specifically bind complex structures such as bacterial cells, thus they can be used to develop rapid diagnostic arrays. In addition, phage-displayed peptides can functionalize surface with less-steric hindrance than antibodies, thus allowing a higher binding avidity for the target per surface unit. In fact, on the same surface unit, a greater number of peptides and with a more correct orientation can be patterned in comparison to antibodies. Furthermore, the nature of the bioreceptor peptide holds potential utilization for development against any bacterium, virus or toxin to which a corresponding phage could be affinity-selected for. Therefore, different peptides could be isolated specifically binding to isolated proteins, enzyme or inorganic material, as well as to different microbial species, thus with the same microsystem different targets might be detected, by performing parallel several different assays in real-time, within the same miniaturized substrate, in a single run. Through the use of different labels in parallel, such as different specific peptides, multiple tests could be simultaneously performed on the same micro-array, so that standardizing data from multiple separate experiments is unnecessary and truly meaningful comparisons can be made.

5. Conclusions

The results of this research demonstrate developmental proof-of-concept biosensor probes for *P. aeruginosa*, based on an affinity-selected recombinant filamentous phage clone from random peptide libraries. 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 clinical-based diagnostics and possibly biological warfare applications. Furthermore, the nature of the bioreceptor layer holds potential utilization for development against any bacterium, virus or toxin to which a corresponding phage could be affinity-selected for. Therefore, other potential markets include food monitoring, research and industrial use.

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