

Display of expression products of cDNA libraries on phage surfaces

A versatile screening system for selective isolation of genes by specific gene-product/ligand interaction

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Techniques for cloning cDNAs from bacteriophage libraries immobilised on solid supports are well established. However, these techniques do not allow selective enrichment of clones expressing proteins of interest. Screening of cDNA libraries would be simplified if the proteins encoded by cDNAs could be expressed on the surface of phage. Phage carrying genes encoding proteins for which a ligand is available can be selected directly by affinity interaction [Cramer, R. & Suter, M. (1993) *Gene (Amst.)* 137, 69–75]. The expression products from a cDNA library from *Aspergillus fumigatus* have been displayed on the surface of the filamentous phage M13 and screened for gene products binding to human serum IgE. The physical linkage of cDNA-encoded proteins to the genetic information required for their production, achieved by exploiting the high-affinity interaction of the Jun and Fos leucine zippers, allows screening of up to 1×10^{10} independent clones in 50- μ l aliquots applied to a well of a microtiter plate coated with the ligand. Phage displaying IgE-binding proteins were selectively enriched 10^5 – 10^6 -fold over non-specific phage after six rounds of growth and selection. The apparent molecular mass of the proteins selected from the cDNA library was in the range 20–40 kDa. Restriction enzyme analysis and preliminary sequence determination of 12 selected inserts revealed different sequences. The ability of the proteins to bind to human serum IgE was corroborated by enzyme-linked immunosorbent assay and by Western-blot analysis. The developed cloning strategy allows isolation of cDNAs encoding proteins for which a ligand is available and circumvents immobilisation of the libraries on solid-phase supports which hamper selective enrichment of clones expressing the desired protein.

The advantage of filamentous phage display cloning over conventional cloning lies in the physical linkage of each protein to the genetic material that encodes it, which is accomplished by fusion of proteins to a phage coat protein [1, 2]. When phagemid-harboring *Escherichia coli* cells are infected with helper phage, the phagemid genome is replicated and packaged into phage particles [3]. As phagemid is extruded from the cell [4], fusion protein is incorporated into the surface coat, along with some copies of the wild-type coat protein encoded by the helper phage. The result is a mixture of wild-type helper phage and recombinant phagemid particles that carry the fusion protein and contain the DNA coding for the cloned protein. Phage that display both wild-type coat protein and fusion protein are infective. Therefore, the recombinant genome that encodes a binding protein may be readily isolated from large libraries by successive rounds of phage growth and selection on surfaces coated with a ligand [5]. The gene III (gIII) coat protein located at the tip of the filamentous phage M13 is responsible

for the attachment of phage to the bacterial F pilus [6] and has been used to display peptide epitopes [7–9], proteins [10–12] and immunoglobulin fragments [13–16] on the surface of phage. Phage expressing fusion proteins of interest were isolated from large display libraries by selection with either antigens [17, 18], antibodies [19], or other ligands [12, 20, 21]. Therefore, filamentous phage systems appear attractive for expression of products of cDNAs. Cloning systems designed to express gene products on the surface of phage have proven to be useful for the display and screening of functional homodimeric or heterodimeric proteins [11, 12, 21, 22]. Unfortunately, these systems do not allow the construction of cDNA expression libraries because translational stop codons present at the 3' end of non-translated regions of eukaryotic mRNA [23] prevent construction of fusion proteins N-terminal to the gIII gene product, while fusions to the membrane-bound C-terminus of gIII [24] would hamper the incorporation of the fusion protein into the phage coat. To covalently link the expressed cDNA product to the phage surface, a strategy based on the phagemid pComb3 [25] and the ability of the leucine zippers Jun and Fos to associate with each other [26, 27] was developed.

A modified gIII product, obtained by fusing the Jun leucine zipper flanked by cysteine residues N-terminal to the viral coat protein, was used to capture expressed cDNA prod-

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Abbreviations. *asp f* Ia, gene encoding the *Aspergillus fumigatus* allergen I; *Asp f* Ia, *A. fumigatus* allergen I; gIII, gene III product of bacteriophage M13.

ucts decorated with the Fos leucine zipper flanked by cysteine residues. The gIII–Jun fusion protein is incorporated into phage particles during phage morphogenesis providing a covalent link to the phage surface for random recombinant cDNA products decorated with the Fos leucine zipper by Jun–Fos heterodimerisation and disulphide bond formation. The resulting phagemid, pJuFo [19], allows expression and display of cDNA libraries on phage surfaces and application of the powerful screening technology based on biopanning procedures used for other filamentous phage systems [11, 12, 25]. In this study, we report the application of pJuFo to directly select a wide variety of cDNAs from a library constructed using mRNA from *Aspergillus fumigatus*, a fungus known to produce a complex pattern of allergenic proteins recognised by IgE antibodies from sera of sensitised patients [28].

MATERIALS AND METHODS

Strains, plasmids and reagents

A. fumigatus strain AF 102 (ATCC 42202) was kindly supplied by Dr V. P. Kurup (Medical College of Wisconsin, Milwaukee, WI). The *E. coli* strains XL1-Blue [29] and M15 [30] were also used. The phagemid pJuFo [19] is a derivative of pComb3 [25] designed to display cDNA gene products on the surface of phage M13 (Fig. 1A). Plasmid pDS56 [31] was used for high-level expression of cloned genes as fusion proteins with six adjacent histidine residues allowing a one-step purification of the proteins using immobilised metal-ion affinity chromatography [32, 33]. Helper phage VCSM13 (*kan^r*) was from Stratagene. DNA-modifying enzymes were purchased from Boehringer Mannheim and were used as recommended by the manufacturer. Chemicals were of the highest available grade and were obtained from Fluka or Sigma unless otherwise specified.

Construction of a cDNA library and screening of library expression products displayed on phage surfaces

For the construction of an *A. fumigatus* cDNA library, 4 µg pJuFo vector [19] was digested with *Xba*I and *Kpn*I; the mixture was subsequently treated with calf intestine phosphatase [34]. 2 µg cDNA inserts, prepared from a previously constructed λ -ZapII expression library [35] by *Xba*I and *Kpn*I digestion of isolated DNA, was ligated to the cleaved vector. The ligation mixture was used to transform *E. coli* XL1-Blue cells by electroporation [36] and a phage surface expression library was generated by helper phage superinfection as published previously [25]. Five independent cDNA expression libraries, differing in size due to variations of the efficiency of the electroporation procedure, were generated. The previously described panning procedure [8, 25] was modified to enrich for phage expressing proteins of interest on their surface (Fig. 1B). Samples of 100 µl monoclonal mouse anti-human IgE mAb TN-142 (0.1 µg µl⁻¹) were coated onto polystyrene microtiter wells overnight at 4°C. Free sites were blocked with 150 mM NaCl, 50 mM sodium phosphate, pH 7.4 (NaCl/P_i) containing 2% (mass/vol.) skimmed milk powder for 1 h at 37°C and washed extensively. Sera from individuals sensitised to *A. fumigatus* (100 µl) were added to each well and plates were incubated at 37°C for 2 h. After washing three times with NaCl/P_i containing 0.5% Tween-20 and three times with NaCl/P_i, a spe-

cific IgE-ligand surface, depleted in interacting and cross-reacting antibodies of other isotypes present in the polyclonal sera, was obtained. 50 µl of the phage library with the highest size (approximately 10¹¹ colony-forming units) was added to a well, and the plate was incubated for 2 h at 37°C. Phage were removed and the well was washed ten times with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20. Phage were eluted by the addition of 100 µl 0.1 M HCl, pH 2.2 (adjusted with solid glycine), 1 mg · ml⁻¹ BSA (buffer A), removed, and neutralised with 6 µl 2 M Tris base. Eluted phage were used to infect 2 ml exponentially growing *E. coli* XL1-Blue cells to prepare phage for a further round of panning as described [19, 25, see above].

Isolation and analysis of phage displaying allergenic proteins

Cultures of phage-transduced bacteria were prepared in 100 ml super broth medium [25] containing 12.5 µg · ml⁻¹ tetracycline and 100 µg · ml⁻¹ ampicillin. Cultures were grown with shaking (250 rpm) at 37°C until an A₆₀₀ value of 0.5 was reached. At this point, helper phage VCSM13 (1 × 10¹² plaque-forming units) were added and the culture was shaken for an additional 2 h. At this time point, 70 µg · ml⁻¹ kanamycin was added and the culture was incubated overnight at 37°C. Phage supernatant (titre 5–10 × 10¹¹ transducing units · ml⁻¹) was prepared by centrifugation (9000 × g for 15 min at 4°C). Phage were precipitated by addition of 4% (mass/vol.) polyethylene glycol 8000 and 3% (mass/vol.) NaCl for 1 h at 4°C followed by centrifugation (9000 × g for 15 min at 4°C). Phage pellets were suspended in 2 ml NaCl/P_i and stored at –20°C.

Enzyme-linked immunoabsorbant assay (ELISA)

The specific binding of human serum IgE, isolated from individuals sensitised to *A. fumigatus*, to phage displaying different *A. fumigatus* allergenic proteins was analysed as described previously [19]. Briefly, phage samples (10¹² transducing units) serially diluted twofold in NaCl/P_i, pH 8.0, were coated overnight at 4°C to Maxisorp polystyrene microtiter plates (Nunc). After washing, the remaining free sites were blocked at 37°C for 1 h with 5% (mass/vol.) non-fat dry milk powder in NaCl/P_i, pH 7.4 (buffer B). The plates were incubated with human sera diluted 1:5 in buffer B containing 5% Tween 20 (buffer C) at 37°C for 2 h. Mouse anti-human IgE mAb TN-142 in buffer C was used as second antibody and was detected with alkaline-phosphatase-conjugated goat anti-mouse IgG (TAGO) as described [37]. Plates were washed between each step with three rinses of 0.5% Tween-20 in NaCl/P_i, pH 8.0, followed by three rinses of NaCl/P_i, pH 7.4. Absorbance readings were measured at 405 nm after a suitable time period [38] with a Molecular Devices Reader (Menlo Park, CA).

High-level production of recombinant proteins

A single-step subcloning of DNA isolated from *E. coli* cells harbouring the replicative form of phagemids scoring positive in ELISA into plasmid pDS56 [31] (Fig. 1C) allows high level production of hexahistidine-tagged fusion proteins which facilitate the purification of recombinant proteins by Ni²⁺-chelate affinity chromatography [32, 33]. *E. coli* M15 cells containing the repressor plasmid pRep4 [31] and plasmid pDS56 with the cloned insert of interest were grown in

1:12×TY medium [34] until an A_{600} value of 0.8 was obtained, induced with 2 mM isopropyl β -D-thiogalactopyranoside, lysed and processed as described [31] to obtain recombinant proteins of high purity.

IgE immunoblot analysis of *A. fumigatus* allergens

Proteins were separated on a SDS/polyacrylamide gradient gel (12.5–20% polyacrylamide) and transferred to a Hybond-ELC nitrocellulose membrane (Amersham) using a Multiphor II Electrophoretic Transfer Unit (Pharmacia) at 60 mA for 2 h [39]. Western blots were developed using the ECL system (Amersham) according to the manufacturer's recommendations. Patient sera were diluted 1:10 and used as 'first antibody'. Mouse anti-human IgE mAb TN-142 was used as the second antibody with detection using horseradish-peroxidase-conjugated goat anti-mouse Ig (TAGO).

RESULTS

Phage surface display of cDNA expression products

We have used phagemid pJuFo [19] (Fig. 1A) to display the proteins encoded in a cDNA library from *A. fumigatus* on the surface of filamentous phage. cDNA expression libraries can be constructed using the unique *Bgl*III, *Xba*I and *Kpn*I restriction sites of the phagemid (Fig. 1A). Five independent cDNA expression libraries were constructed. The size of the libraries varied between 2×10^7 and 3×10^9 independent clones recovered as ampicillin resistant colonies; this corresponds to an efficiency of transformation between 3×10^6 and $5 \times 10^8/\mu\text{g}$ DNA of the ligation mixture. As it was shown that phage expressing small peptide epitopes can be purified from mixtures of other phage using antisera or antibodies [7, 8], we attempted to purify phage displaying allergens from an *A. fumigatus* expression library subcloned into the pJuFo phagemid using sera of patients allergic to the mold.

Selective enrichment of clones binding to human IgE

The phage library, typically 1×10^{11} transducing units in 50 μl , was added to a single well of a microtiter plate that was previously coated with IgE from serum of allergic individuals (Fig. 1B). After washing, adherent phage were eluted from the well by the addition of buffer A, titered and used to infect *E. coli* cells to prepare phage for a further round of phage growth and selection. Phage enrichment was monitored without screening by following the yield of phage eluted from each round of panning (Table 1) and selectivity of the enrichment was demonstrated by immunological criteria. Clones derived from phage transduction for each round of panning were grown in liquid culture; phage were produced by superinfection with helper phage, purified and analysed for binding to human IgE antibodies by specific ELISA (Fig. 2). After six rounds of affinity selection, all tested clones yielded phage displaying proteins recognised by human IgE (Table 1). The method has been shown to allow specific sorting of phage encoding the *asp f* I/a gene [35] with a monoclonal antibody raised against the *Asp f* I/a protein [40] at a frequency of *asp f* I/a-encoding phage as low as one phage in a population of 1×10^7 in the starting mixture [19]. Thus, rare phage coding for a specific protein could be enriched from large populations using the appropriate ligand for the gene product.

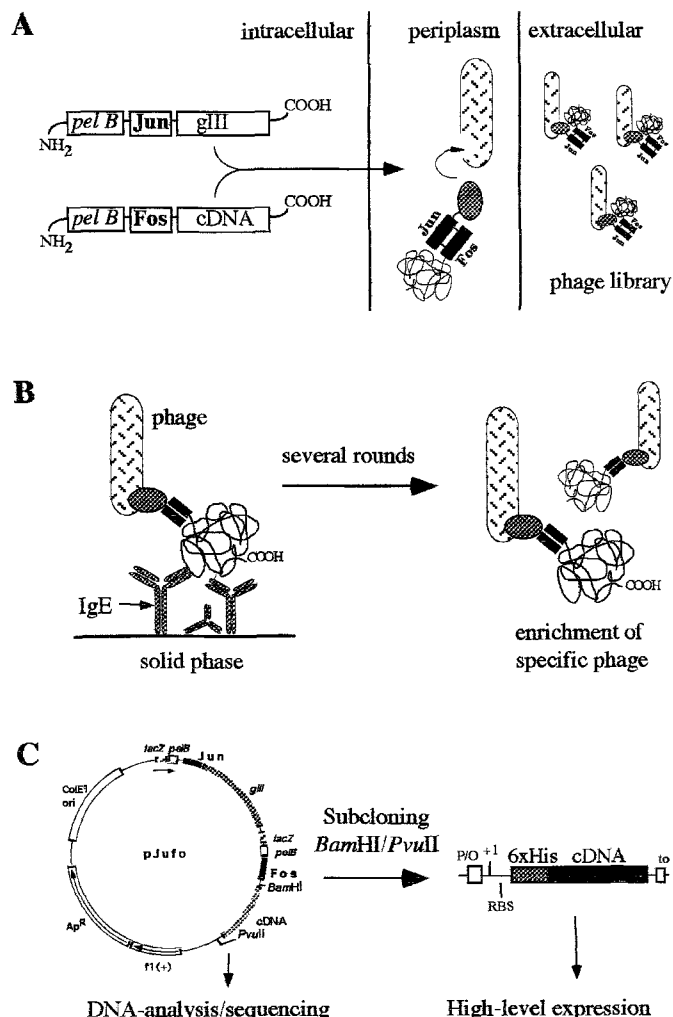


Fig. 1. Generation of phage surface display libraries, enrichment of phage displaying desired products and high-level expression of the isolated genes. (A) Essential elements of the pJuFo phagemid. The Jun leucine zipper flanked by cysteine residues is expressed and secreted as an N-terminal fusion to the gIII gene product of the phagemid. The Fos leucine zipper flanked by cysteine residues is used to produce Fos–cDNA fusions secreted via the *pelB* leader and captured by the Jun leucine zipper. cDNA expression libraries can be constructed using the unique *Bgl*III, *Xba*I and *Kpn*I restriction sites of the phagemid. (B) Enrichment of phage displaying cDNA products on the surface. A ligand coated to a solid-phase surface is used to capture phage expressing cDNA-encoded products by ligand/product interaction. Phage that bind specifically to the ligand can be isolated after consecutive rounds of phage growth and adsorption. (C) High-level expression of isolated cDNAs. A single-step subcloning into plasmid pDS56 allows high-level production of hexahistidine-tagged fusion proteins that facilitate purification of the recombinant protein by Ni^{2+} -chelate affinity chromatography.

Screening of a cDNA library from *A. fumigatus* with human serum IgE antibodies as ligand, yielded a large population of phage expressing IgE-binding proteins (Table 1). The phage population enriched by ligand/product interaction is likely to contain phage encoding different allergenic proteins. To test this assumption, DNA from cells producing proteins scoring positively in ELISA with human IgE antibodies was extracted and the size of the insert was determined by restriction enzyme analysis and agarose gel electrophoresis. At least 12 different restriction patterns were obtained (data not

Table 1. Enrichment of phage binding to human serum IgE antibodies from individuals sensitised to *A. fumigatus*. Phage displaying cDNA products from an *A. fumigatus* expression library were applied to a single well of a microtiter plate coated with human serum IgE. After adsorption and extensive washing, bound phage were eluted and used to infect *E. coli* for a further round of phage growth and selection. Single colonies from each round of panning were grown in liquid cultures, phage induced and purified phage were coated directly onto microtiter plates and tested for IgE-binding affinity by an IgE-specific ELISA. The enrichment factor is the yield (%) of phage from each round of panning [yield = (number of phage eluted) × 100 / (number of phage applied)]. IgE-binding phage represents phage binding to IgE/number of phage tested.

Round of panning	Phage applied	Phage eluted	Enrichment factor	IgE-binding phage
1	1.6×10^{11}	9.0×10^3	5.6×10^{-6}	0/10
2	8.0×10^{10}	2.0×10^4	2.5×10^{-5}	0/10
3	8.0×10^{10}	3.0×10^4	3.8×10^{-5}	0/10
4	8.0×10^{10}	1.5×10^5	1.9×10^{-4}	1/10
5	1.6×10^{11}	3.6×10^6	2.3×10^{-3}	8/10
6	2.2×10^{11}	8.7×10^6	4.0×10^{-3}	10/10

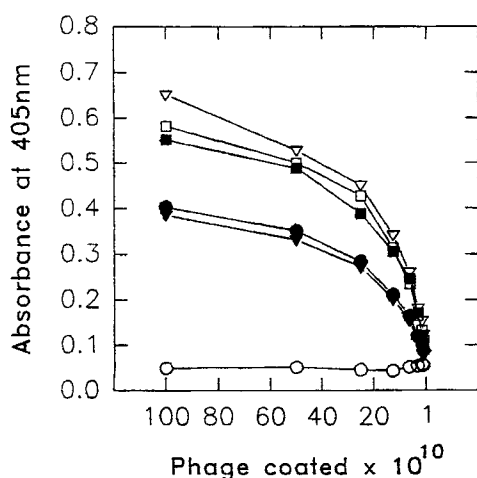


Fig. 2. Antigen-specific ELISA. Binding of human serum IgE from *A. fumigatus* sensitised individuals to phage displaying different *A. fumigatus* allergenic molecules (▽, □, ■, ●, ▼) and phage pJuFo (○) as control. The clone with closed circles (●) corresponds to the earlier described rAsp f I/a [35] as judged from its 5' nucleotide sequence (data not shown).

shown). A sample of five clones differing in insert size and restriction pattern were chosen for further investigation. Dissociation between phage and expressed cDNA products can readily be achieved by SDS/PAGE under reducing conditions. However, for large-scale protein production and purification the inserts were subcloned into the expression vector pDS56 [31] (Fig. 1C). *E. coli* M15 transformants produced 40–90 mg recombinant proteins/l culture after induction with isopropyl β -D-thiogalactopyranoside and the proteins were readily purified by metal chelate affinity chromatography in a single step. These proteins were analysed by SDS/polyacrylamide gradient gels (12.5–20% polyacrylamide) and were demonstrated to be IgE-binding proteins by Western-blot analysis (Fig. 3). The apparent molecular mass of the proteins was in the range 20–40 kDa. Restriction enzyme analysis and nucleotide sequence determination at the 5' and 3' ends of the inserts revealed that they are unlikely to encode related proteins (data not shown). Thus, genes can be selectively enriched from cDNA expression libraries displayed on the surfaces of phage through interaction of the gene product with a specific ligand.

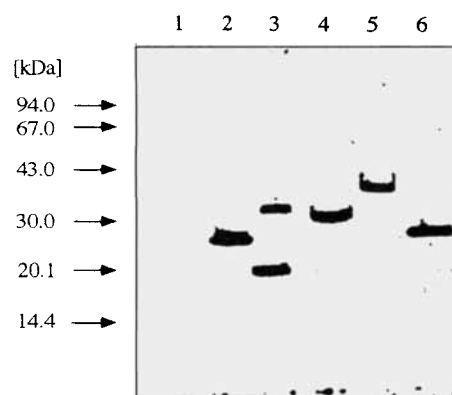


Fig. 3. IgE immunoblot of *A. fumigatus* allergens. 1.5 μ g each protein was separated on a SDS/polyacrylamide gradient gel (12.5–20% polyacrylamide) and transferred to a nitrocellulose membrane. Binding of serum IgE from *A. fumigatus* sensitised individuals to the allergens was assayed by Western blotting. Marker proteins (lane 1) served as negative controls. The positions of the markers are indicated (kDa). Protein in lane 3 corresponds to the earlier described rAsp f I/a present in monomeric and dimeric form [35].

DISCUSSION

Previously we have demonstrated that phage displaying the *asp f* I/a gene [35] can be enriched from a phage mixture using a monoclonal antibody raised against the *Asp f* I/a protein as a ligand [19]. In this study, we show that phage encoding allergenic proteins displayed on phage surfaces can be selectively isolated from a cDNA expression library using human serum IgE antibodies as ligand. Due to the complex pattern of allergenic proteins produced by *A. fumigatus* [28, 41] and to the polyclonal nature of the serum IgE antibodies, a population of phage encoding different allergenic proteins was obtained. Obvious advantages of the pJuFo surface display compared to conventional solid-phase based screening systems are the ability to access very large libraries with a powerful enrichment technique, and the possibility to select for genes physically linked to their gene product as secreted proteins. Secreted proteins are believed to assume a native-like structure [42] and phage enrichment in a liquid phase avoids denaturation effects due to adsorption of proteins to nitrocellulose membranes [43]. The latter two points are specially relevant for the screening of allergenic proteins, where IgE antibodies recognise conformation-dependent discontinuous epitopes [44, 45].

The demonstration that proteins encoded in a cDNA library can be displayed on the surfaces of phage and screened by ligand/product interaction has implications beyond the isolation of allergenic proteins. Antibody fragments [1, 2, 13–17, 25] and enzymes [19] expressed on the surface of filamentous phage are generally biologically active. Proteins expressed on phage surfaces need to be secreted into the periplasmic space of *E. coli*, a process known to favour protein refolding [42], and may therefore be more adequate than proteins expressed in other systems when complex ligands that bind to complicated topological structures are used for screening. pJuFo has been used successfully to display intracellular proteins like mouse dihydrofolate reductase and cyclin-dependent kinases from man and hamster (Cramer, R. and Jaussi, R., unpublished results). Thus, the system is not limited to naturally secreted proteins. The largest protein we have detected displayed on phage surfaces using pJuFo has a molecular mass of approximately 72 kDa (Cramer, R., unpublished results).

Other cloning systems designed to express gene products on the surfaces of phage have proven to be extremely powerful for display and screening of functional homodimeric or heterodimeric proteins [1, 10, 11, 14, 15]. However, these systems do not allow the construction of cDNA expression libraries because translational stop codons present at the 3' end of non-translated regions of eukaryotic mRNA prevent the construction of fusion proteins N-terminal to the gIII gene product of the phage. The proposed cloning system circumvents this problem using the Jun leucine zipper fused N-terminal to the gIII coat protein of the phage to capture cDNA gene products decorated with the Fos leucine zipper. The cysteine residues flanking both leucine zippers provide a covalent link between the gene product and the genetic information required for its production avoiding inter-phage product exchanges. The pJuFo phagemid can, in principle, be used to isolate the gene encoding any protein for which a specific polyclonal or monoclonal antibody is available. In order to use the pJuFo phagemid for screening purposes, only cDNA and a ligand to screen for affinity interaction are required. Therefore, a wide range of receptor/ligand schemes can be envisaged. For example, phage encoding proteins involved in DNA recognition (helix-turn-helix and zinc-finger motifs) [46] could be isolated with DNA as ligand or directly with zinc ions immobilised on a support derivatised with a chelating agent. The latter strategy has been successfully used to select phage encoding antibodies binding to metal ions from semi-synthetic combinatorial antibody libraries [20]. The feasibility of accessing DNA-binding proteins with a phage display system has been demonstrated by selecting zinc-finger proteins with altered DNA-binding specificity by random mutagenesis of the amino acids involved in DNA recognition [12]. Furthermore, pJuFo will allow the screening of cDNA expression libraries with any ligand, such as drugs, heavy metals or anti-metabolites, as a tool for the discovery of functionally important proteins or targets of potential industrial interest.

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