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Rapid isolation of novel FK506 binding proteins from multiple organisms using gDNA and cDNA T7 phage display

Andrew M. Piggott †, Alison M. Kriegel, Robert D. Willows, Peter Karuso *

Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia

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ABSTRACT

Reverse chemical proteomics using T7 phage display is a powerful technique for identifying cellular receptors of biologically active small molecules. However, to date this method has generally been limited to cDNA libraries constructed from mRNA isolated from eukaryotes. In this paper, we describe the construction of the first prokaryotic T7 phage display libraries from randomly digested *Pseudomonas stutzeri* and *Vibrio fischeri* gDNA, as well as a plant cDNA library from *Arabidopsis thaliana*. We also describe the use of T7 phage display to identify novel proteins from environmental DNA samples using biotinylated FK506 as a model affinity probe.

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1. Introduction

The identification of cellular receptors for natural products and drugs is a prerequisite for understanding their modes of action and has become an area of intense research interest in recent years.[1,2] Affinity purification (forward chemical proteomics), whereby a homogenized cellular extract is probed with the ligand immobilized on a solid support, is by far the most commonly used method, but suffers from several drawbacks, most notably that the large dynamic range of protein concentrations in a typical proteome means that the most abundant binding protein is often isolated instead of the most avid binder.[3,4] An alternative method of identifying cellular receptors for biologically active molecules is reverse chemical proteomics, which utilizes a 'genotype–phenotype link' to physically join proteins to their encoding genes.[1,2] The main advantage of this system is that it is iterative in nature, allowing amplification of low copy-number proteins and hence ensuring that the most avid (and hence most biologically relevant) binding partner, rather than the most abundant binding partner, is isolated.

The most widely used method of reverse chemical proteomics is phage display,[5] whereby a transcriptome is cloned into the genome of a bacteriophage such that the encoded proteins are 'displayed' on the surface of the phage particles fused to the coat proteins. In this manner, each displayed protein generally behaves as if it was free in solution, yet can be replicated many times by simply infecting a susceptible bacterial host. The most common form of phage display utilizes bacteriophage M13, principally for the display of random peptides or antibodies.[6] The display of eukaryotic cDNA libraries is less common as most of the M13 systems are N-terminal display and thus the translational stop signals obtained if poly(A)-primed mRNA is used results in no protein being displayed. The only C-terminal display system available for M13 is for protein VI, but it has not proved popular.[7] In contrast, the pJuFo-system, which uses N-terminal display of the Jun-gene product on M13 phage protein III and tagging the cDNA library with the Fos-gene, has been widely utilized.[8] In both systems, the foreign DNA is inserted after its fusion partner (C-terminal fusions), thereby avoiding the stop codon, and correct fusions are only required at one end of the inserts. Both the pJuFo and pVI systems give a monovalent display of the foreign protein, which is advantageous for the isolation of strong binding partners but disadvantageous for the isolation of moderate or weak binding partners. In this respect, we are unaware of any M13 phage display system being used to isolate the binding partners for small molecules such as drugs or natural products, which tend to have only moderate binding affinities with their protein targets when compared to antibody–protein interactions.

T7 phage display does has a proven track record in the isolation of protein targets for natural products and drugs, including FK506,[9] doxorubicin[10] and kahalalide F.[11] The T7 system has several characteristics that make it an attractive vector for biopanning studies.

* Corresponding author. Tel.: +61 2 9850 8290; fax: +61 2 9850 8313.
E-mail address: peter.karuso@mq.edu.au (P. Karuso).
† Present address: Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Qld 4072, Australia.

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T7 is robust, easy to grow and replicates more quickly than lambda or filamentous phages, decreasing the amount of time required to perform each round of selection. Unlike filamentous phages, T7 particles are released by cell lysis, and are not secreted through the bacterial cell membrane, allowing a greater variety of proteins to be displayed on the surface of the phages, with no selective advantage being conferred to hydrophobic proteins or proteins inherently associated with membranes. The lytic process also releases cell-based cofactors that may be required for interactions between the displayed protein and the immobilized ligand. The gp10 expression system is naturally C-terminal, eliminating the stop codon problem with cDNA libraries, and the valency can be adjusted from 0.1 to 415 copies of the foreign gene product per phage particle by modifying the promoter region upstream of the gp10 gene. The size of the foreign protein can also be quite large, with proteins of up to 1200 amino acids having been expressed in a functional form.[12] However, all T7 cDNA libraries to date have been constructed from eukaryotic mRNA, presumably due to the difficulty in handling prokaryotic mRNA.

Despite its successes, a common criticism of phage-displayed cDNA libraries is that the method cannot reproduce any post-translational modifications found in many eukaryotic proteins and that proper protein folding may be inhibited by the lack of ER and appropriate chaperones. In addition, proteins may be expressed poorly due to differential codon usage between host and library. Many of these disadvantages could be overcome by the cloning of bacterial gDNA libraries. The so called 'shot-gun phage display' was pioneered by Frykberg's group in Sweden with *Staphylococcus aureus* gDNA.[13,14] Chromosomal DNA is fragmented with ultrasound to generate 100–700 bp fragments, which are electrotransformed into *Escherichia coli* cells that have been infected with R408 helper phage. The M13 phage libraries have up to 10^7 clones and have been used to isolate small binding domains of bacterial proteins such as those that bind to surgical implants.[15] This group has worked exclusively with M13 phage and there have been no examples of gDNA libraries in other phage display systems or attempts to construct libraries that contain a large proportion of intact genes.

Herein, we describe the construction of the first prokaryotic T7 libraries using randomly digested gDNA from *Pseudomonas stutzeri* and *Vibrio fischeri*. The quality of the newly constructed libraries was evaluated using a validated biotin-FK506 probe. In addition, the use of reverse chemical proteomics to identify novel cellular receptors for affinity/activity based probes, and to identify novel proteins from environmental DNA samples, was explored.

2. Results and discussion

2.1. Synthesis of biotin-FK506 probe

In any reverse chemical proteomics study, the quality of the results obtained is largely dependent on the quality of the phage display library used. Therefore, when constructing new libraries, it is useful to have a validated model system to evaluate the performance of each library. FK506 is a macrolide antibiotic isolated from *Streptomyces tsukubaensis*, which exhibits potent immunosuppressive activity.[16] FK506 binds strongly (K_d ~0.4 nM) to its cellular receptor FKBP1a (FKBP12), which is an abundant 12 kDa cytosolic protein.[17] FKBPs are found almost universally across species, both in prokaryotes and eukaryotes, where their primary role may be to catalyze the cis–trans isomerization of proline residues, which has been proposed as a rate-limiting step in protein folding.[18,19] Importantly, FKBPs have been shown to retain the ability to bind FK506, even when displayed on the surface of a T7 phage particle.[9,20] Therefore, FK506 is an ideal pre-validated probe to evaluate the quality of newly constructed T7 phage display libraries from both prokaryotes and eukaryotes.

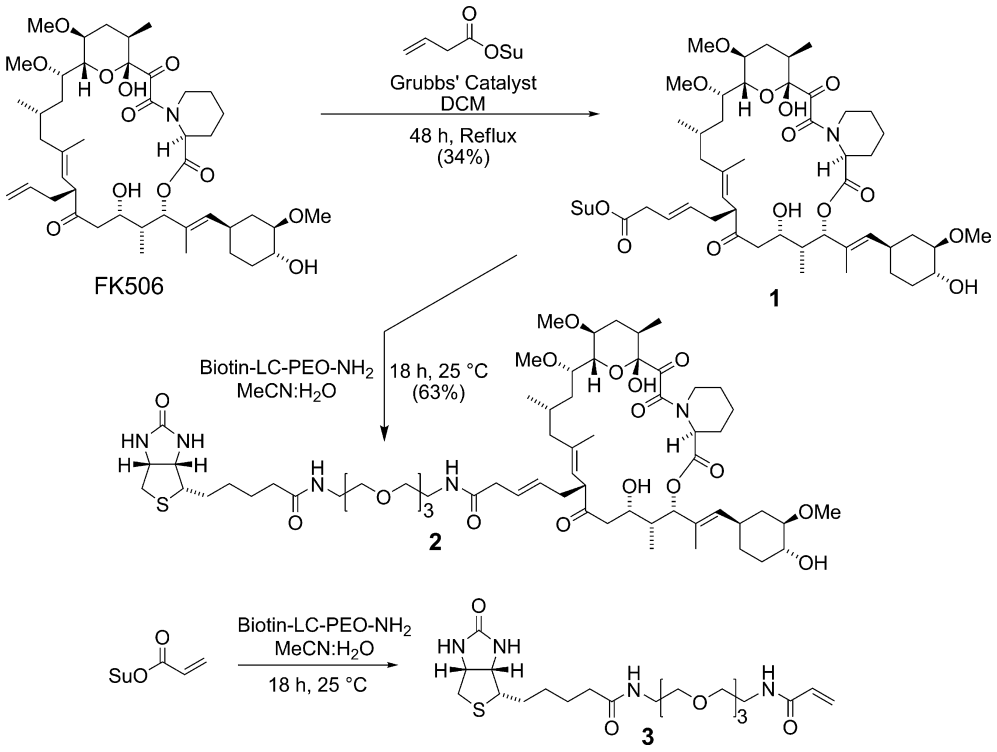
When synthesizing probes for chemical proteomics studies, it is vital that the derivatization process does not alter the biological activity of the molecule and hence the site of derivatization must be chosen carefully.[2] X-ray crystal structures show that FKBP binds to the α,β-diketoamide group of FK506 and that the terminal olefin on the opposite side of the macrocyclic ring points directly away from FKBP when bound.[21] Therefore, the terminal olefin of FK506 is an appropriate site for derivatization. Sche et al.[9] adapted a method described by Schreiber and co-workers[22,23] to attach a biotinylated linker to FK506 via its terminal olefin. This method involved protection of the secondary hydroxyl of FK506 with TMSOTf, conversion of the terminal olefin to an aldehyde with OsO_4/$NaIO_4$ followed by reduction to a primary alcohol with LTEPA, formation of a mixed carbonate with DSC, attachment of a Boc-protected hexanediamine spacer, simultaneous deprotection of the Boc and TMS protecting groups with HF and finally derivatization with commercial Biotin-LC-NHS, yielding biotin-FK506 in seven steps with an overall yield of 19%.

We have developed a shorter and simpler method of synthesizing biotin-FK506 that also retains the double bond of the FK506 side-chain (Scheme 1). Initially, a Grubbs' catalyst-mediated cross metathesis reaction[24] between FK506 and vinylacetic acid NHS was used to activate the terminal olefin, yielding FK506-NHS (**1**). A large excess of vinylacetic acid NHS relative to FK506 was used to minimize the formation of FK506 dimer. The active ester was then reacted with Biotin-LC-PEO-NH_2, yielding biotin-FK506 (**2**) in 21% overall yield in just two steps. The low yielding cross metathesis reaction could conceivably be improved by using the more reactive second generation Grubbs' catalyst.[25] A control probe (**3**), containing only the vinyl group of FK506 was constructed by reacting Biotin-LC-PEO-NH_2 with acrylic acid NHS.

2.2. Affinity selection of FK506 binding proteins from human brain cDNA library

A commercially available human Alzheimer brain T7Select10-3 cDNA library was used to evaluate the performance of the biotin-FK506 probe and the affinity selection procedures. Clarified phage lysate was pre-incubated in microtiter plate wells coated with the biotin-acrylate (**3**) control plate to remove non-specific binders and was then transferred to microtiter plate wells coated with biotin-FK506 (**2**) and left to incubate for 4 h. Following incubation, the phage solution was aspirated and the wells washed with buffer. For the first round of selection, a low stringency washing step (3 washes in 10 s) was used to ensure that moderate affinity binders present in low copy numbers were not lost. With each successive round of selection, the proportion of phages displaying the target protein(s) should increase, so the stringency of the washing step (number of washes and washing time) was progressively increased, up to 20 washes in 30 s in Round 6 and then 30 washes in 60 s in Round 7. Finally, phages retained by the affinity support were released by addition of SDS and were used to infect fresh *E. coli* cells to generate a sublibrary for the next round of selection.

The number of phage particles eluted from the plate after each round provides a useful indication of whether the affinity selection has been successful, with a significant increase in titer indicating that selective enrichment of phages capable of binding to the affinity probe has occurred. In this experiment, the phage titer increased significantly after four rounds of selection (Fig. 1A), suggesting that the biotinylated FK506 affinity probe was successful in selectively enriching one or more clones from the cDNA library, while the titer did not increase significantly from Round 5 onwards, suggesting a consensus had been reached. The slight decrease in titer between Rounds 6 and 7 most likely due to the



Scheme 1. Synthesis of biotin-FK506 (**2**) affinity probe and biotin-acrylate (**3**) control.

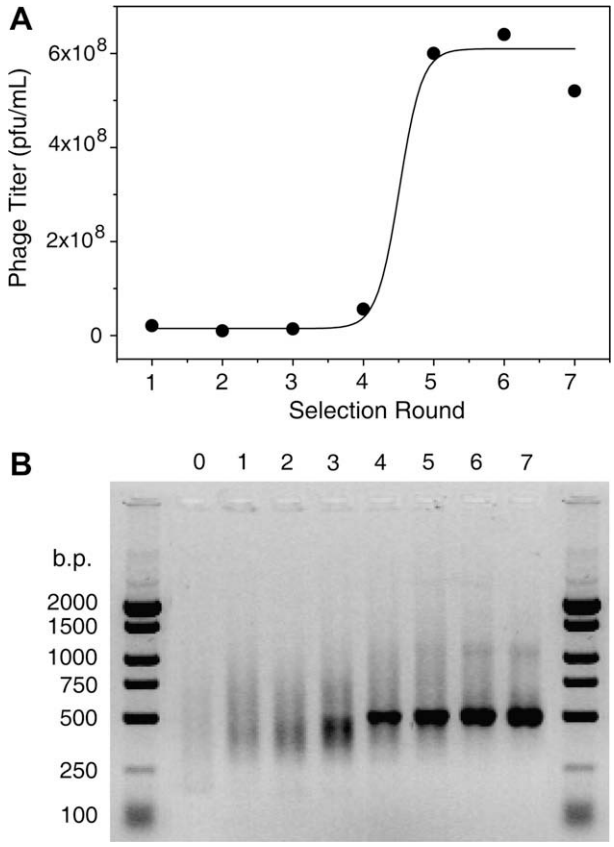


Figure 1. (A) Phage titers and (B) agarose gel electrophoresis of phage DNA inserts amplified by PCR from Alzheimer brain cDNA library (Round 0) and after 1–7 rounds of selection with biotin-FK506 (**2**) immobilized on a NeutrAvidin-coated plate.

markedly increased wash stringency used for Round 7, highlighting the importance of using a low stringency wash for the initial rounds of selection as even the strongest binding phages can be displaced if the washing step is too long.

The DNA inserts from phages surviving each round of selection were amplified by PCR using generic T7 primers and separated by gel electrophoresis (Fig. 1B). The appearance of a band around 500 bp after four rounds of selection, coinciding with the sigmoidal increase in phage titer, is also indicative of selective enrichment of one or more clones from the cDNA library. Twenty-four random plaques were selected from the Round 4 sublibrary and their DNA inserts were amplified by PCR and separated by gel electrophoresis (Fig. 2). An aliquot of the amplified DNA was also digested with a frequent base cutter (*Hin*fI) to generate a unique fingerprint of each insert. Seven plaques were found to have DNA inserts of ~500 bp and have similar DNA fingerprints (Fig. 2; A2, C2, F2, H2, C3, F3 and D4). The DNA from one of these plaques (A2) was sequenced and was found to contain the C-terminal fragment (aa 45-142) of human FKBP2.

The isolation of only FKBP2 from human Alzheimer brain is particularly noteworthy as previous T7 phage display studies using an analogous biotinylated FK506 probe and normal human brain libraries[9,20] have only isolated the predominant human isoform of FKBP (FKBP1a). FKBP2 binds FK506 less strongly than FKBP1a in vitro (K_d ~55 nM)[26] and hence would not be expected to be the most avid binder. While the FK506 binding domains of FKBP1a and FKBP2 are identical, the latter has a leader sequence of 32 amino acids (Fig. 3A). As this leader sequence (and a further 10 residues) was absent in the phage-displayed clones isolated, it is possible that, in this experiment, the FKBP2 clones had a higher affinity for FK506 than the FKBP1a clones. A specific interaction between biotin-FK506 and the FKBP2 phage was confirmed by performing a binding study, whereby a single T7 clone displaying the FKBP2 fragment showed 1000-fold higher affinity for FK506 (**2**) derivatized microtiter plates than for

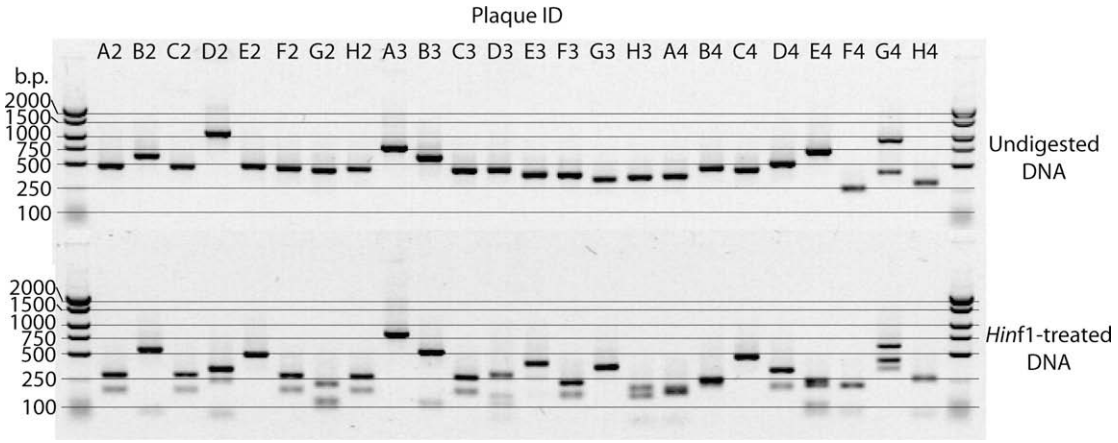


Figure 2. Agarose gel electrophoresis of PCR products obtained from Alzheimer brain individual plaques after four rounds of selection with biotin-FK506 (**2**) immobilized on a NeutrAvidin-coated plate. The DNA inserts were also digested with HinfI to produce unique DNA fingerprints of each clone.

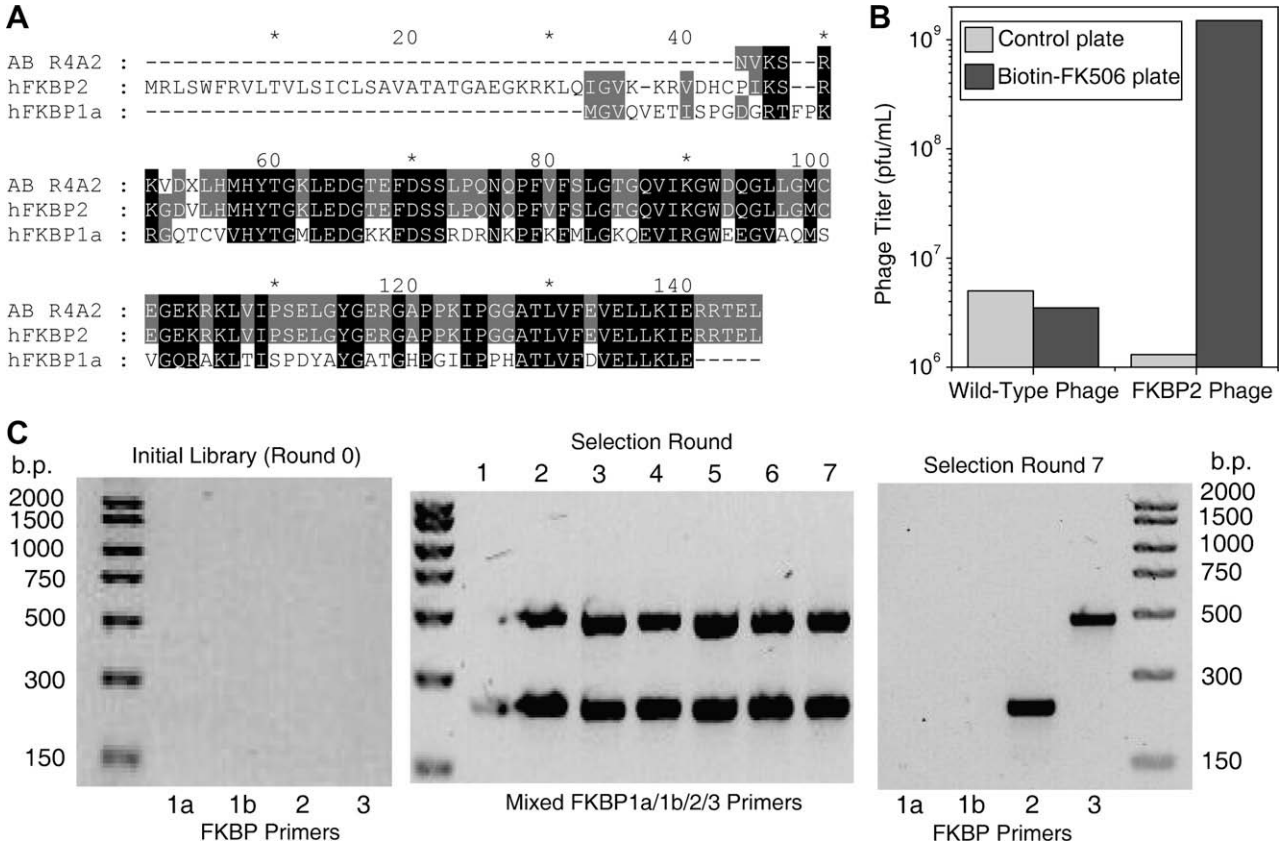


Figure 3. (A) Sequence alignment of protein encoded by human Alzheimer brain Round 4 plaque A2, human FKBP2 (Swiss-Prot P26885) and human FKBP1a (Swiss-Prot P62942). (B) Binding study comparing affinity of wild-type and FKBP2 phages for biotin-acrylate (**3**) and biotin-FK506 (**2**). (C) Agarose gel electrophoresis of the Alzheimer brain cDNA library probed with specific primers for FKBP1a, FKBP1b, FKBP2 and FKBP3 (left panel); PCR products obtained from Alzheimer brain cDNA library for Rounds 1–7 obtained with a mixed set of primers containing specific up- and down-primers for FKBP1a. FKBP1b, FKBP2 and FKBP3 (middle panel); and after seven rounds of selection against biotin-FK506 (**2**) using primers specific for human FKBP1a, FKBP1b, FKBP2 and FKBP3 (right panel).

biotin-acrylate (**3**) plates (Fig. 3B). PCR was performed on the initial Alzheimer brain cDNA library using primers specific for human FKBP1a, FKBP1b, FKBP2 and FKBP3 to determine whether these genes could be detected in the library (Fig. 3C). None of the three genes could be amplified from the initial T7 cDNA library (Fig. 3C left panel) but could be detected after selection with FK506. Even from the first round of selection, a faint band for FKBP1a/1b is visible (slightly smaller size than FKBP2) but by Round 2 only FKBP2 and FKBP3 could be detected (Fig. 3C; middle panel). After seven rounds of selection with biotin-FK506 (**2**), only the primers for FKBP2 and FKBP3 resulted in amplification of a gene (Fig. 3C; right panel).

Interestingly, inhibitors of FKBP have been shown to possess neuroprotective and neuroregenerative activity[19,27] and hence FKBPs may play a role in the progression of neurodegenerative diseases such as Alzheimer's disease. FKBP2 is also known to localize in the lumen of the endoplasmic reticulum,[28] which is where the folding of membrane and secreted proteins occurs, as

well as being a major site of intracellular calcium storage. Alzheimer's disease has been linked to both accelerated production or delayed clearance of protein aggregates[29] and neuronal calcium mishandling.[30] These links between Alzheimer's disease and FKBPs may result in higher concentration of FKBP2 in Alzheimer's diseased brains.

2.3. Affinity selection of FK506 binding proteins from *Arabidopsis thaliana* cDNA library

Having confirmed that our biotin-FK506 probe and panning protocol can efficiently rescue FKBP clones from a large and complex commercial library, we set about constructing and testing our own library from cDNA isolated from the plant *A. thaliana*. *A. thaliana*, which is seen as the model eudicot species, is the first plant to have its genome completely sequenced and, as such, a T7 phage display library would have numerous potential uses. Therefore, an *A. thaliana* cDNA library was constructed using subtractive cloning on Oligotex beads.[31] Total RNA was extracted from frozen plant material from three life cycle stages (seedling, mature plant and seeding stage) and all tissue types. The normalized mRNA library was cloned into Novagen T7Select10-3b phage vector to yield 7.6×10^6 distinct clones. The diversity of the library was assessed by picking 40 random clones and PCR amplification/restriction digestion as for the bacterial gDNA libraries. Distinct amplicons were obtained for only 19 of the 40 plaques, with 3 amplicons the expected size of wild-type phage. The 21 plaques that didn't give amplicons gave smears ranging from 200 to 800 bp suggesting false priming and/or mixed templates. The lack of a distinct amplicon was a common occurrence when screening plaques from the *A. thaliana* library and may be due to long inserts which are difficult to amplify.

The *A. thaliana* cDNA library was subjected to five rounds of selection using biotin-FK506 (**2**) as an affinity probe in an analogous manner to the Alzheimer brain cDNA library. Phages surviving each round of selection were titered (Fig. 4A) and their DNA inserts were amplified by PCR using generic T7 primers (Fig. 4B). The overall phage titer increased progressively between Rounds 3 and 5, suggesting selective enrichment of phages capable of binding to the affinity support was occurring. This corresponded to the appearance of an intense band around ~700 bp in the DNA gel starting from Round 3. Thirty random plaques were selected from the Round 5 sublibrary and their DNA inserts were amplified by PCR and separated by gel electrophoresis (Supplementary data, Fig. S3). An aliquot of the amplified DNA was also digested with a frequent base cutter (*Hin*fI) to generate a unique fingerprint of each insert. Electrophoresis of the PCR products from randomly selected clones showed that, after only five rounds of biopanning, 60% of the isolated clones contained similar genes, 20% contained no insert (150 bp band) and the final 20% were random clones. Sequencing of three of the dominant clones showed that all three contained different FKBP genes (Fig. 5). Clone B1 was identical to the previously described *A. thaliana* FKBP1a (Swiss-Prot Q8LGG0) but contained a 9 amino acid leader sequence. Clone F9 was almost identical to *A. thaliana* FKBP1a, containing two conservative mutations (D54E and V118I). Clone A10, however, was quite different, with only 59% sequence identity and 72% similarity to *A. thaliana* FKBP1a.

The rapid isolation of three different FKBPs from *A. thaliana*, including one previously undescribed homolog, highlights the power of reverse chemical proteomics using T7 phage display. By selecting appropriate affinity/activity based probes, it is possible to quickly and conveniently identify novel cellular receptors with the demonstrated ability to bind small molecule targets, thereby providing protein/ligand pairs for rational drug design or for probing cellular biology.

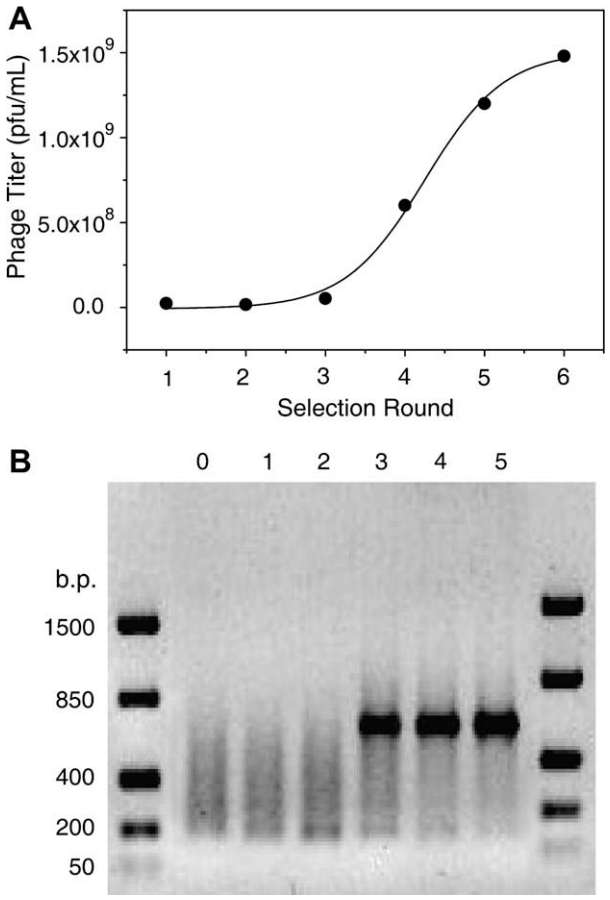


Figure 4. (A) Phage titers and (B) agarose gel electrophoresis of phage DNA inserts amplified by PCR from *Arabidopsis thaliana* cDNA library (Round 0) and after 1–5 rounds of selection with biotin-FK506 (2) immobilized on a NeutrAvidin-coated plate.

2.4. Affinity selection of FK506 binding proteins from *P. stutzeri* randomly digested gDNA library

Following the construction and validation of our plant cDNA library, our attention turned to the construction of prokaryotic T7 libraries using randomly digested gDNA. To date, T7 gene fragment libraries have been limited to cDNA libraries constructed from the mRNA of eukaryotes. The absence of prokaryotic T7 cDNA libraries is almost certainly due to the difficulty in handling prokaryotic mRNA, which has a very short lifetime and has little or no polyadenylation. However, given that the size of most prokaryotic genomes is modest, that prokaryotic DNA is uninterrupted by introns and that T7 has the ability to accommodate large fragments of DNA, we concluded that it would be feasible to construct a prokaryotic T7 phage display library by random digestion of gDNA.

In order to construct a representative library from a prokaryotic organism, the method used to fragment the DNA must be selected carefully to increase the probability of obtaining fusion proteins that are both in-frame and of sufficient size for selection to be successful. Two methods that could be used are random partial digestion with a 4-base recognition restriction enzymes and ultrasonic shearing of DNA as used for M13 library construction.[13,14] Statistically, random full digestion with *Sau*3A1 will yield fragments of the genome separated at ~256 bp intervals, while ultrasonic fragmentation will yield fragments that depend strongly on the amount of power used. Ultrasonic fragmentation also requires further processing and addition of linkers to the sheared DNA for successful ligation. Because of the relative ease with which a random digested

library can be constructed versus a library made with sheared DNA we decided to use partial digestion with *Sau*3A1 for our initial prokaryotic library. A partial *Sau*3A1 digest of gDNA from *P. stutzeri* was size-selected before cloning into T7 phage. The library constructed contained a total diversity was 1.94×10^5 distinct clones, with only 2% containing no insert. The size range of inserted DNA fragments in the remaining phage was between 30 and 1800 bp, with a median insert size of 400 bp.

The *P. stutzeri* gDNA library was subjected to five rounds of selection using biotin-FK506 (**2**) as an affinity probe in an analogous manner to the human Alzheimer brain cDNA library described earlier. Phages surviving each round of selection were titered (Fig. 6A) and their DNA inserts were amplified by PCR using generic T7 primers (Fig. 6B). The overall phage titer increased dramatically between Rounds 3 and 5, suggesting selective enrichment of phages capable of binding to the affinity support was occurring. This corresponded to the appearance of an intense band around 800 bp in the DNA gel starting from Round 4. Sixteen random plaques were selected from the Round 4 sublibrary and their DNA inserts were amplified by PCR and separated by gel electrophoresis (Supplementary data, Fig. S4). An aliquot of the amplified DNA was also digested with a frequent base cutter (*Hin*fI) to generate a unique fingerprint of each insert. Eight plaques (B10, C10, G10, H10, D11, E11, F11, G11) had DNA inserts of ~800 bp and also had identical DNA fingerprints. The DNA from one of these plaques (E11) was sequenced and was found to contain a novel FKBP with 85% sequence identity and 92% similarity to the known *P. stutzeri* FKBP (Fig. 7). Four identical plaques (D10, F10, B11 and C11) were found to contain a short DNA fragment of the gene encoding a haloacid dehydrogenase, while the remaining two plaques (A10 and E10) either contain a very short DNA fragment or no DNA insert at all (wild-type).

In addition to evaluating the quality of the *P. stutzeri* gDNA T7 library, the results of the selection with biotin-FK506 demonstrate that reverse chemical proteomics can be used for the rapid isolation and identification of novel proteins from environmental DNA samples. In addition, our results show that it should be possible to rapidly isolate the protein binding partners for new antibiotics or natural products and thereby identify new antibiotic targets.

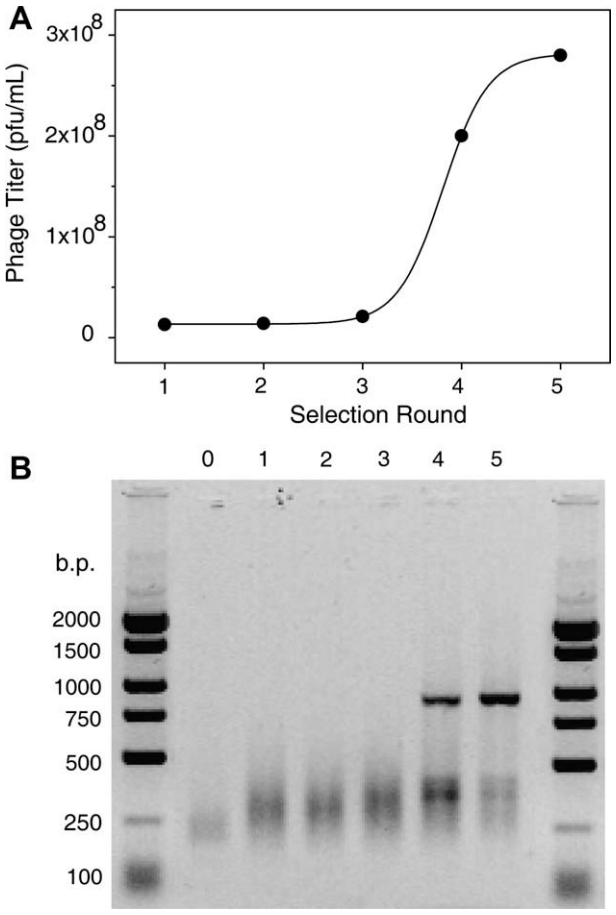


Figure 6. (A) Phage titers and (B) agarose gel electrophoresis of phage DNA inserts amplified by PCR from *Pseudomonas stutzeri* gDNA library (Round 0) and after 1–5 rounds of selection with biotin-FK506 (**2**) immobilized on a NeutrAvidin-coated plate.

2.5. Affinity selection of FK506 binding proteins from *V. fischeri* randomly digested gDNA library

Vibrio species have been reported to contain large superintegrons and have a high percentage of hypothetical and unidentified genes.[32] *V. fischeri* is genetically quite similar to human pathogens (e.g., *V. cholera*) with the notable exception of virulence factors such as CT or RTX. In addition, *V. fischeri* is a model organism for the study of quorum sensing in bacteria, in which this phenomenon was first discovered.[33] Therefore, this organism is a potentially rich source of novel bacterial gene products that have particular relevance to human pathogens and hence we considered it to be a good target for creating a T7 phage display library that could be used to isolate potentially new antibiotic targets. *V. fischeri* gDNA was partially digested and a T7 library constructed as described for *P. stutzeri* to yield a total diversity of 4.13×10^5 distinct clones. All of 46 randomly picked plaques contained DNA inserts of between 20 and 1350 bp with a median insert size of 170 bp and an average size of 275 bp.

The *V. fischeri* gDNA library was subjected to five rounds of selection using biotin-FK506 (**2**) as an affinity probe in an analogous manner to the human *P. stutzeri* library. Phages surviving each round of selection were titered (Fig. 8A) and their DNA inserts were amplified by PCR using generic T7 primers (Fig. 8B). After five

```
                          *         20        *         40        *         60
Arab_R5A10  :  ----SKKKKKGGGKNQFFKPGNGPKPAPGQTFPVLCPGFGKKGDLSQNFGITRDKGKNLFSFQIG  :  61
Arab_R5B1   :  -SERQREDIEMGVEKQVIRPGNGPKPAPGQTVTVHCTGFGKDGDLSQKFWSTKDEGQKPFSFQIG  :  64
Arab_R5F9   :  NSAEERR-IEMGVEKQVIRPGNGPKPAPGQTVTVHCTGFGKDGDLSQKFWSTKEEGQKPFSFQIG  :  64
Arab_FKBP12 :  ----------MGVEKQVIRPGNGPKPAPGQTVTVHCTGFGKDGDLSQKFWSTKDEGQKPFSFQIG  :  55

                     *         80        *        100        *        120
Arab_R5A10  :  KGAGIKGGEKGFFEMQFEKFAPWRGSSNYPYGPGGFPAGGTHPNSFLNFKIKLLTVQK  :  119
Arab_R5B1   :  KGAVIKGWDEGVIGMQIGEVARLRCSSDYAYGAGGFPAWGIQPNSVLDFEIEVLSVQ-  :  121
Arab_R5F9   :  KGAVIKGWDEGVIGMQIGEVARLRCSSDYAYGAGGFPAWGIQPNSVLDFEIEILSVQ-  :  121
Arab_FKBP12 :  KGAVIKGWDEGVIGMQIGEVARLRCSSDYAYGAGGFPAWGIQPNSVLDFEIEVLSVQ-  :  112
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Figure 5. Sequence alignment of proteins encoded by *Arabidopsis thaliana* plaques B1, F9 and A10 with known *A. thaliana* FKBP1a (Swiss-Prot Q8LGG0).

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                          *        20         *        40         *        60
Rnd 4 Plaque E11 : ------------------------------------------------------------AVAQG :   5
P. stutzeri FKBP : MRLRHQRLAPAIALVGLVLAGCDSQTSVKLDTPAQKASYGIGLNMGKSLAQEGMDDLDSKAVAQG :  65

                       *        80         *       100         *       120         *
Rnd 4 Plaque E11 : IDDALTKQEQKLTDEELMEAFAFLQTRAEERMVEMNKEAVAAGEKFLADNAKREGVKTTESGLQY :  70
P. stutzeri FKBP : IEDAIGNKEQKLTDEQLMEAFTFLQTRAEERMAEMNAKAVEAGKKFLEENAKREGVKTTESGLQY : 130

                              140         *       160         *       180         *
Rnd 4 Plaque E11 : EVIKQADGAQPTAEDVVTVHYEGSLVDGTVFDSSIKRGSPIDLPVGGVIPGWVEGLQLMHVGEKY : 135
P. stutzeri FKBP : EVVKSAEGRQPTENDVVTVHYEGSLTDGTVFDSSIKRGSPIDLPVGGVIPGWVEGLQLMHVGEKF : 195

                           200         *       220         *       240         *
Rnd 4 Plaque E11 : KLYIPSELAYGEQSPSPLIPANSVLIFELELLGIKGDESQAPAEEVAPEAAEPATEQ : 192
P. stutzeri FKBP : KLYIPSELAYGEQSPSPLIPANSVLVFDLELLDIKGDEAEQPAEAAEQQ-------- : 244
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Figure 7. Sequence alignment of protein encoded by *Pseudomonas stutzeri* Round 4 plaque E11 with known *P. stutzeri* FKBP (Swiss-Prot A4VN69).

rounds of selection, the overall phage titer had not increased, suggesting no enrichment of phages had occurred. In addition, no distinct bands had appeared in the DNA gel. A further two rounds of selection (6 and 7) were performed, with no improvement. Sixteen random plaques were selected from the Round 4 sublibrary and their DNA inserts were amplified by PCR and separated by gel electrophoresis (Supplementary data, Fig. S5). An aliquot of the amplified DNA was also digested with a frequent base cutter (*Hin*fI) to generate a unique fingerprint of each insert. The plaques isolated

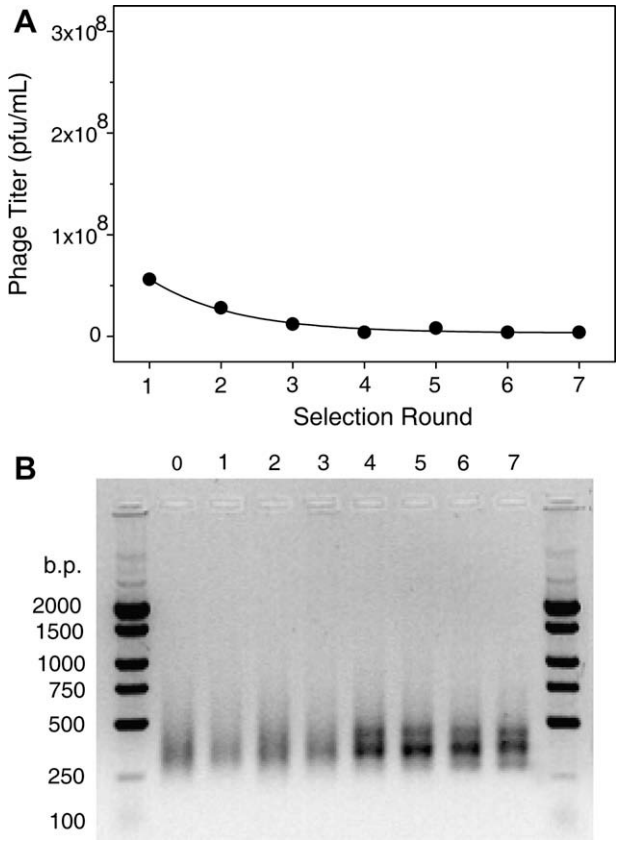

Figure 8. (A) Phage titers (on the same *y*-axis scale as for *P. stutzeri*) and (B) agarose gel electrophoresis of phage DNA inserts amplified by PCR from *Vibrio fischeri* gDNA library (Round 0) and after 1–5 rounds of selection with biotin-FK506 (**2**) immobilized on a NeutrAvidin-coated plate.

were highly variable in length and none had identical DNA fingerprints. This confirms that the selection was unsuccessful, with no selective enrichment of phages capable of binding to the affinity support occurring.

The *V. fischeri* genome (GI:197333880) does contain at least 3 FKBP genes that have homologous binding sites to human FKBP1a and hence the biotin-FK506 probe should have been capable of rescuing these clones. However, it should be noted that the median size of the *V. fischeri* library was only 170 bp (56 aa), compared to 400 bp (133 aa) for *P. stutzeri*, and hence it is possible that the DNA fragments were too small to accommodate a full-length FKBP gene. In addition, each of the 3 FKBP encoding genes in *V. fischeri* has a *Sau*3A1 site within the coding region, reducing the odds of obtaining a full-length gene. Given the small average size of the inserts in this library this suggests that a FKBP sequence which is long enough to code for a functional FKBP domain is not present in the library.

3. Conclusion

In this paper, we have described the construction of the first prokaryotic gDNA T7 phage display libraries, and have demonstrated how these libraries can be exploited, using immobilized FK506, to discover new FKBP genes. We have shown that our biotin-FK506 probe, synthesized concisely via a cross metathesis reaction without deletion or protection of any functional group of the natural product, can be used to assess the quality of phage display libraries. We found that affinity selections using biotin-FK506 on a commercial Alzheimer's brain T7 cDNA library yielded a C-terminal fragment of FKBP2, rather than the more abundant and higher affinity FKBP1a. T7 libraries constructed from *A. thaliana* cDNA and *P. stutzeri* gDNA led to the isolation of two new FKBP genes. In contrast, a T7 library constructed from *V. fischeri* gDNA did not yield any binding partners when screened under identical conditions. It is clear that a sigmoidal increase in phage titer and appearance of distinct bands on the gel of the PCR products are requisites for a successful reverse chemical proteomics experiment for the isolation of natural product binding proteins from genomic libraries. We suggest that, because FKBP proteins are ubiquitous, FK506 can be used as a convenient probe to confirm the quality of phage libraries derived from DNA or RNA or to compare different panning protocols. This method has recently been used by Sugawara and co-workers to develop panning protocols for PEGA resin and quartz-crystal microbalance.[34,35] Biopanning gDNA libraries of

human pathogens with biotinylated antibiotics should prove to be a rapid and safe method for the identification of new antibiotic targets and in mechanism of action studies for a range of small molecules that affect bacterial growth and reproduction. We are currently investigating this possibility.

4. Experimental

4.1. General experimental details

4.1.1. Equipment

NMR spectra were recorded in 5 mm Pyrex tubes (Wilmad, USA) on either a DPX-400 400 MHz or DRX-600 K 600 MHz spectrometer (Bruker, Germany) using residual undeuterated solvent as an internal reference. High resolution ESI mass spectrometry was performed by Prof. Gary Willett (University of New South Wales, Australia) on an APEXII Fourier transform ion cyclotron resonance spectrometer (Bruker, Germany). Low resolution ESI mass spectrometry was performed on a Quattro-II triple quadrupole spectrometer (Fisons Instruments, USA). HPLC was performed using a 600E multisolvent delivery system and a 490 programmable multiwavelength detector (Waters, USA). Water was purified using a Milli-Q Ultrapure Water Purification System (Millipore, USA). Bacterial cultures were incubated in a heated orbital shaker (Thermoline Scientific, Australia). Solutions were centrifuged with a 6K15 refrigerated centrifuge (Sigma, Germany). DNA was amplified with a GeneAmp PCR System 2400 Thermocycler (Perkin Elmer, USA). DNA sequencing was performed by Mr. Paul Worden (Macquarie University DNA Analysis Facility) using an ABI Prism 377 Sequencer (Applied Biosystems, USA). Agarose gel electrophoresis was performed using a Mini-Sub Cell GT system (Bio-Rad, USA) and gels were visualized with a ChemiImager digital imaging system (Alpha Innotech, USA).

4.1.2. Materials

Dichloromethane was distilled under nitrogen from calcium hydride. Chloroform was distilled under nitrogen from phosphorus pentoxide. DMSO was dried over microwave-activated 4 Å molecular sieves. Ethanol, isopropanol and light petroleum were glass distilled. *N*-hydroxysuccinimide was recrystallized from ethanol. All other solvents and reagents were used without further purification.

T7Select10-3 human Alzheimer brain cDNA library and *E. coli* strain BLT5615 were obtained from Novagen Inc. (USA). T7SelectUp (5′-GGAGCTGTCGTATTCCAGTC-3′), T7SelectDown (5′-AACCCC TCAAGACCCGTTTA-3′), FKBP1a forward (5′-GGGATGCTTGAAGATG GAAA-3′), FKBP1a reverse (5′-GAAGACGAGAGTGGCATGTG-3′), FKBP1b forward (5′-ACAGGAATGCTCCAAAATGG-3′), FKBP1b reverse (5′- CAGCTCCACGTCAAAGATGA-3′), FKBP2 forward (5′-GAAGCTGG AAGATGGGACAG-3′) and FKBP3 reverse (5′-ATTTTGAGCAGCTC CACCTC-3′), FKBP3 forward (5′-GAAGCTGGAAGATGGGACAG-3′) and FKBP3 reverse (5′-ATTTTGAGCAGCTCCACCTC-3′) primers were synthesized by Sigma-Genosys (Australia). *Taq* DNA polymerase and QIAquick PCR purification kits were obtained from QIAGEN (USA). *Hin*fI restriction endonuclease and NEB buffer 2 were obtained from New England Biolabs Inc. (USA).

4.2. Synthesis of biotin-FK506 probe

4.2.1. Vinylacetic acid

A solution of vinylacetic acid (1.0 g, 12 mmol), NHS (1.4 g, 12 mmol) and DCC (2.5 g, 12 mmol) in anhydrous chloroform (25 mL) was stirred at room temperature for 18 h, during which time a white precipitate formed. The precipitated DCU was filtered off and the filtrate was reduced to dryness in vacuo to give a pale-yellow oil. To remove the remaining traces of DCU, the oil was redissolved in DCM, loaded onto a short silica column packed in DCM and eluted with DCM. The column eluate was reduced to dryness in vacuo, yielding vinylacetic acid NHS as a colorless oil (1.9 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 5.87–5.97 (tdd, 1H, J = 16.9, 10.2, 6.7 Hz), 5.32 (dtd, 1H, J = 17.0, 1.5, 1.1 Hz), 5.30 (dtd, 1H, J = 10.2, 1.5, 1.1 Hz), 3.39 (ddd, 2H, J = 6.7, 1.5, 0.1 Hz), 2.83 (br s, 4H, 2 × NCH_2).

4.2.2. FK506-NHS (1)

A solution of FK506 (5.0 mg, 6.2 μmol) and vinylacetic acid NHS (20 mg, 110 μmol) in DCM (10 mL) was sparged with argon for 15 min. Grubbs' catalyst generation I (1.0 mg, 1.2 μmol) was then added and the solution was refluxed for 24 h under argon. A second portion of catalyst (1.0 mg, 1.2 μmol) was added and refluxing was continued for a further 24 h. The reaction mixture was then reduced to dryness in vacuo, redissolved in ethyl acetate (5 mL) and passed through a plug of silica, eluting with ethyl acetate, to remove ruthenium salts. The eluate was further purified by HPLC (Alltech, Econosphere silica column, 250 × 4.6 mm, 5 μm, 1 mL/min, gradient from 15% to 40% isopropanol/light petroleum), resulting in two UV active peaks (230 nm) eluting after 24 and 26 min in a 1:2 ratio. These two peaks were collected, combined and reduced to dryness under a stream of nitrogen, yielding a mixture of cis and trans isomers of **1** as a white powder (2.0 mg, 34%). Mass spectrum (HRESI+) *m/z*: 981.4911 ([M+Na]$^+$), calcd for $C_{50}H_{74}N_2O_{16}Na^+$ 981.4931.

4.2.3. Biotin-FK506 (2)

A solution of **1** (1.19 mg, 1.25 μmol) and EZ-Link Biotin-LC-PEO-Amine (0.519 mg, 1.25 μmol; Pierce Chemical Company, Rockford, IL.) in aqueous acetonitrile (90%; 5 mL) was stirred at room temperature for 18 h. The crude reaction mixture was then purified by HPLC (Phenomenex Gemini C_{18} column, 250 × 4.6 mm, 5 μm, 1 mL/min, gradient from 50% to 100% acetonitrile/water over 45 min), resulting in a peak (230 nm) eluting after 11 min. This peak was collected and the solvent removed under a stream of nitrogen, yielding **2** as a white solid (1.0 mg, 63%). See Supplementary data Figs. S1–S2 for NMR spectra. Mass spectrum (HRESI+) *m/z*: 653.8398 ([M+2Na]$^{2+}$), calcd for $C_{64}H_{103}N_5O_{18}SNa_2^{2+}$ 653.8402.

4.2.4. Acrylic acid NHS

A solution of DCC (5.6 g, 27 mmol) in chloroform (25 mL) was added dropwise to a solution of acrylic acid (1.9 g, 27 mmol) and NHS (3.1 g, 27 mmol) in chloroform (50 mL) over 1 h and the reaction mixture stirred at room temperature for a further 18 h. The solution was then reduced to dryness in vacuo and the residue taken up in DCM and loaded onto a silica column equilibrated with DCM. The column was developed with DCM and six fractions were collected. The first three fractions were combined (TLC) and reduced to dryness in vacuo to give a waxy solid, while the last three fractions, containing DCU, were discarded. The waxy solid was recrystallized from absolute ethanol yielding acrylic acid NHS as a white crystalline solid (2.6 g, 57%). Mp 65–66 °C. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 6.70 (dd, 1H, J = 17.3, 0.9 Hz), 6.30 (dd, 1H, J = 17.3, 10.7 Hz), 6.16 (dd, 1H, J = 10.7, 0.9 Hz), 2.86 (s, 4H).

4.2.5. Biotin-acrylate (3)

A solution of acrylic acid NHS (4.1 mg, 24 μmol) and EZ-Link Biotin-LC-PEO-Amine (10 mg, 24 μmol; Pierce Chemical Company, Rockford, IL.) in aqueous acetonitrile (90%; 5 mL) was stirred at room temperature for 18 h. The solvent was then removed under a stream of nitrogen, yielding crude **3** as a colorless gum, which was used without further purification. Mass spectrum (ESI+) *m/z:* 495 ([M+Na]$^+$), 473 ([M+H]$^+$).

4.3. Preparation of biotin-FK506-derivatized microtiter plates

A solution of **2** (1 mg) in DMSO (1 mL) was diluted to 20 mL with PBS and an aliquot (100 μL) was added to each well of a Neutravidin-coated polystyrene microtiter plate (Pierce Reacti-Bind). The plate was left to stand at 4 °C overnight before the solutions were aspirated and each well was washed with PBS (3 × 250 μL). A second plate was derivatized with the control compound 3 in a similar fashion. All microtiter plate wells were pre-incubated with PBS (250 μL) for 1 h at room temperature then washed with PBS (3 × 250 μL) immediately before use.

4.4. Construction of T7 phage libraries

4.4.1. Construction of *A. thaliana* cDNA library

A. thaliana var. Columbia seeds were germinated on Gamborg's B-5 Basal Medium (Sigma–Aldrich, St Louis MI) and grown to different growth stages in soil under green house conditions. Whole plants, including roots, were selected at the seedling stage, at flowering and after setting seed. A total of 25 g of plant material were frozen in liquid nitrogen and ground to powder. Total RNA was isolated from 1 g of mixed tissue (RNeasy midi-kit, Qiagen, Valencia, CA) using 4 mL of Buffer RLT. Oligotex mRNA kits (Qiagen, Valencia, CA) were used to isolate mRNA from 0.5 mg of total RNA with 60 μL of Oligotex beads eluted in 200 μL of Buffer OEB before being freeze-dried.

Oligotex-cDNA was prepared from *A. thaliana* mRNA as per the manufacturers instructions (Qiagen, Valencia, CA). The Oligotex-cDNA was pelleted and resuspended in 100 μL of 10 (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl) with 100 μg $dA_{30}G_{10}$ (1 mg/mL) and then incubated at 65 °C for 5 min and then at 37 °C for 10 min. The supernatant was removed (and retained for use in Round 2) and the blocked Oligotex-cDNA was resuspended in 100 μL hybridization buffer including 5 μg mRNA and 6 μg of oligo dT (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% SDS) and incubated at 55 °C for 30 min. Oligo dT prevents mRNA from annealing to the remaining free oligo (dT) residues on the Oligotex beads not blocked by the $dA_{30}G_{10}$. The sample was centrifuged and supernatant removed. This supernatant represents Round 1 subtracted mRNA, which was stored on ice. Oligotex-cDNA beads were then regenerated for reuse as follows. The used beads were resuspended in 400 μL TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) and incubated at 90 °C for 3 min to denature the cDNA/mRNA complex then chilled on ice for 1 min. The sample was centrifuged and supernatant removed (mRNA previously hybridized to Oligotex-cDNA with sequences expressed in common between the two sample sources). The pellet was washed with 200 μL TE and then resuspended in $dA_{30}G_{10}$-containing supernatant (saved from above) and the entire subtractive hybridization process is repeated using Round 1 subtracted mRNA to yield Round 2 subtracted/normalized mRNA. Two rounds of subtraction were usually performed to normalize the mRNA sample prior to cDNA synthesis.

SDS was removed from subtracted/normalized mRNA (100 μL) with 1 vol. of 0.3 M KCl, left on ice for 1 h. The sample was spun and the supernatant retained. The pellet was washed with 0.3 M KCl and supernatant was combined with the original. Normalized mRNA was freeze-dried and then resuspended in 10 μL RNase-free water, with the concentration assumed to be between 0.5 and 1.0 μg mRNA. A 20-μL reaction, including 1 μg normalized mRNA and 0.5 μg oligo dT primer (Promega, Madison WI), was incubated at 70 °C for 10 min and then quickly chilled on ice water. First strand and second strand cDNA synthesis was performed with the Novagen OrientExpress cDNA Cloning System (EMD Biosciences, Gibbstown, NJ) according to the manufacturer's instructions. The cDNA ends were blunt-ended with T4 DNA polymerase before 100 pmol Directional *Eco*R1/*Hin*dIII linkers were ligated with 6 Weiss units of T4 DNA ligase at 16 °C for 16 h. The linker-fused cDNA was restriction digested with *Hin*dIII followed by *Eco*R1 digestion. The cDNA was phenol-chloroform extracted followed by precipitation and then stored at −20 °C.

Uncut DNA (EMD Biosceicnes, Gibbstown, NJ) was digested with *Eco*RI and *Hin*dIII (Promega). Ligation reactions with normalized and digested *A. thaliana* cDNA were performed with 1:1, 1:3 and 1:5 vector/insert ratios using T4 DNA ligase (Roche Applied Science, Penzberg, Germany) at 16 °C for 16 h. The ligation mixtures were packaged in vitro using T7Select packaging extract and titered as described in the T7Select System Manual.[36] The diversity of the library was determined by PCR amplification of 40 randomly selected plaques with the primers T7SelectUP and T7SelectDOWN. PCR products were digested with *Hin*f1 to produce DNA fingerprints of each clone.

4.4.2. Construction of *P. stutzeri* randomly digested gDNA library

The *P. stutzeri* gDNA T7 phage display library was constructed using the Novagen T7Select system (EMD Biosciences, Gibbstown, NJ). Uncut T7Select10-3b DNA was digested with *Bam*H1 (Promega, Madison, WI) and then treated with shrimp alkaline phosphatase (Promega, Madison, WI). The digested vector was purified by phenol/chloroform extraction and then exchanged into TE buffer (10 mM Tris, 1 mM EDTA, pH 8) using a Sephacryl S400 gel filtration column (GE Healthcare, Upsala Sweden). *P. stutzeri* gDNA (environmental isolate from PCB contaminated soil, Balmain Power Station, Sydney, Australia) was partially digested with *Sau*3A1 (Promega, Madison, WI) for 15 min. to give an average of 500–1500 bp fragments. DNA fragments smaller than ~300 bp were removed using Sephacryl S400 gel filtration.

Ligation reactions were performed with 1:1, 1:3 and 1:5 vector/insert ratios using T4 DNA ligase (Roche Applied Science, Penzberg. Germany) at 16 °C for 16 h. The ligation mixtures were packaged in vitro using T7Select packaging extract and titered as described in the T7Select System Manual,[36] yielding a total of 1.94×10^5 distinct clones within the library. The total packaged library was amplified by infecting mid-log phase *E. coli* BLT5403 cells, plated out on LB-amp agar plates at a density of approximately 40 phage/cm^2, so that individual plaques were obtained. Phages were extracted from the plates with phage extraction buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$), with sufficient volume to cover the plate, for 4 h at 4 °C. The extraction buffer was collected, 0.5 mL chloroform added, centrifuged (3000*g*; 5 min) and the clarified supernatant retained and stored at 4 °C for short term use or at −80 °C after addition of 0.1 vol of 80% glycerol for long-term storage.

4.4.3. Construction of *V. fischeri* randomly digested gDNA library

The *V. fischeri* gDNA T7 phage display library was constructed using the Novagen T7Select system as described for *P. stutzeri*. The diversity of the library was determined by PCR amplification of 46 randomly selected plaques with the primers T7SelectUP and T7SelectDOWN. PCR products were digested with *Hin*f1 to produce DNA fingerprints of each clone. The total packaged library was amplified by infecting mid-log phase *E. coli* BLT5403 cells, plated out on LB-amp agar plates at a density of approximately 40 phage/cm^2, so that individual plaques were obtained. Phages were extracted from the plates with phage extraction buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$), with sufficient volume to cover the plate, for 4 h at 4 °C. The extraction buffer was collected, 0.5 mL chloroform added, centrifuged (3000*g*; 5 min) and the clarified supernatant retained and stored at 4 °C for short term use or at −80 °C after addition of 0.1 volumes of

80% glycerol for long-term storage. Aliquots of phage were also stored at −80 °C after precipitation with PEG.

4.5. Procedure for T7 phage library biopanning

Initial T7 phage libraries and subsequent sublibraries were amplified using *E. coli* BLT5615 as described in the T7Select System Manual.[36] Briefly, log phase *E. coli* BLT5615 cells (which express the T7 coat protein gp10A under the control of a lacUV5 promoter) were infected with the Alzheimer brain cDNA phage library and then incubated at 37 °C until lysis was observed. Tween-20 (1% in PBS) was added to each clarified T7 phage lysate to give a final concentration of 0.01% and an aliquot of each lysate (100 μL) was added to the control plate derivatized with biotin-acrylate (**3**). After incubating for 2 h at room temperature to remove non-specific binders, the lysates were then transferred to the biotin-FK506 (**2**) derivatized plate and were left to incubate for 4 h. Finally, each well of the plate was washed with phage wash buffer (PWB) (3 × 250 μL over 10 s) and incubated with SDS (1%; 100 μL) for 30 min at room temperature. The SDS eluates were used to reinfect *E. coli* for the next round of selection. The stringency of the washing step (number of washes and length of time) was increased for each successive round. A small aliquot of the SDS eluate was retained for titering while the remainder was diluted 1:1000 with log phase *E. coli* cells to begin the next round of selection. This procedure was repeated until 5–7 rounds of selection had been completed. The libraries were assessed by picking random clones and digesting the PCR products with *Hinf*1 to produce DNA fingerprints of each clone. See Supplementary data for a detailed description of titering, picking plaques and *Hinf*I fingerprinting.

4.6. Procedure for T7 phage binding study

Aliquots (100 μL) of clarified phage lysate (containing 0.01% Tween-20) from both wild-type phage and a single T7 clone displaying human FKBP2 were transferred to the control plate derivatized with biotin-acrylate (**3**) and to the plate derivatized with biotin-FK506 (**2**). The plates were left to incubate for 2 h at room temperature and were then washed extensively with PWB (5 × 250 μL over 1 min, soak in PWB for 5 min, then 10 × 250 μL over 1 min). Remaining phage were eluted by incubation with SDS (1%; 100 μL) for 30 min at room temperature and then titered.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.039.

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