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Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains

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ABSTRACT

The display of proteins on the surface of phage offers a powerful means of selecting for rare genes encoding proteins with binding activities. Recently we found that antibody heavy and light chain variable (V) domains fused as a single polypeptide chain to a minor coat protein of filamentous phage fd, could be enriched by successive rounds of phage growth and panning with antigen. This allows the selection of antigen-binding domains directly from diverse libraries of V-genes. Now we show that heterodimeric Fab fragments can be assembled on the surface of the phage by linking one chain to the phage coat protein, and secreting the other into the bacterial periplasm. Furthermore by introducing an amber mutation between the antibody chain and the coat protein, we can either display the antibody on phage using *sup*E strains of bacteria, or produce soluble Fab fragment using non-suppressor strains. The use of Fab fragments may offer advantages over single chain Fv fragments for construction of combinatorial libraries.

INTRODUCTION

The fd and M13 phages are members of the Ff filamentous, single-stranded DNA phages that infect male *Escherichia coli* cells. Adsorption to the host sex pili is mediated by a minor coat protein (gene 3 protein: g3p) displayed at one tip of the virion. Each virion appears to contain three copies of g3p (1). The amino-terminal domains, which form knob-like structures extending from the phage particle, are responsible for binding the phage to the F-pilus, whilst the C-terminal region is anchored in the phage coat (2). Peptides have been displayed at the surface of phage by fusion to the N-terminus of g3p (3), and phage with binding activities isolated from random peptide libraries after rounds of growth and panning (4, 5, 6).

Folded proteins have also been displayed as fusions with g3p (7, 8). Thus, genes encoding binding activities can be selected directly by panning of the recombinant phage. For example, rare antigen-binding phage have been selected from diverse repertoires of antibody genes from immunised animals and the stronger binders enriched by panning (9). In principle the use of phage display libraries from naive antibody genes might allow antibodies to be made entirely *in vitro*: higher affinity variants might be made by random mutation of the genes, so by-passing human or animal immunisation (10).

Antibodies are heterodimeric proteins: the heavy and light chain variable (V) domains combine to make the antigen-binding site. For display on the surface of phage we previously linked both domains into a single polypeptide (7, 9), via a flexible polypeptide chain as a single chain Fv fragment (scFv) (11, 12). We now sought to display the antibodies as heterodimeric Fab fragments, to allow the two chains to be readily reassorted in combinatorial libraries (see Discussion). Since in phage assembly, the coat proteins are exported into the bacterial periplasm, and antibody heavy and light chains can be directed into the bacterial periplasm by a signal sequence (13, 14), we decided to link either the heavy or light chain to the phage g3p with a signal sequence, and co-express the complementary chain into the periplasm. Since antibody heavy and light chains fragments are assembled together in the bacterial periplasm (14), the pairings of heavy and light chains of each bacterium should be retained on the surface of the phage.

MATERIALS AND METHODS

E.coli strains

TG1 :K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*/F'*traD*36, *proA*$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15

HB2151 :K12, *ara*, Δ(*lac-pro*), *thi*/F'*proA*$^+$*B*$^+$, *lacI*^{q}*Z*Δ*M*15

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Oligonucleotides

G3FUFO, 5'-CAG TGA ATT CTT ATT AAG ACT CCT TA-T TAC GCA GTA TGT TAG C; G3FUBA, 5'-TGC GAA G-CT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G; VH1BACKAPA, 5'-CAT GAC CAC AGT GCA CAG GT(C/G) (A/C)A(A/G) CTG CAG (C/G)AG TC(A/T) GG; VH1BACK-SFI15, 5'-CAT GCC ATG ACT CGC GGC CCA GCC GG-C CAT GGC C(C/G)A GGT (C/G)(A/C)A (A/G)CT GCA G(C/G)A GTC (A/T)GG; FABNOTFOH, 5'-CCA CGA TTC TGC GGC CGC TGA AGA TTT GGG CTC AAC TTT CTT GTC GAC; FABNOTFOK, 5'-CCA CGA TTC TGC GGC C-GC TGA CTC TCC GCG GTT GAA GCT CTT TGT GAC; MVKBAAPA, 5'-CAC AGT GCA CTC GAC ATT GAG C-TC ACC CAG TCT CCA; MVKBASFI, 5'-CAT GAC CAC GCG GCC CAG CCG GCC ATG GCC GAC ATT GAG CTC ACC CAG TCT CCA; VK3F2NOT, 5'-TTC TGC GGC CGC CCG TTT CAG CTC GAG CTT GGT CCC. Restriction sites are underlined.

Vector constructions

The two vectors used for expression are depicted in Figure 1. The vector fd-tet-DOG1 (Figure 1A) (9) is derived from fd-CAT1 (7) in turn derived from fd-tet (15). fd-tet-DOG1 has *Apa*LI and *Not*I restriction sites (Figure 1C) for cloning antibody genes as fusions to the N-terminus of g3p. The phagemid pHEN1 (Figure 1B) is a derivative of pUC119 (16): the coding region of g3p from fd-tet-DOG1, including signal peptide and cloning sites, was amplified by PCR, using primers G3FUFO and G3FUBA (which contain *Eco*RI and *Hin*dIII sites respectively), and cloned as a *Hin*dIII-*Eco*RI fragment into pUC119. The *Hin*dIII-*Not*I fragment encoding the g3p signal sequence was then replaced by a pelB signal peptide (13) with an internal *Sfi*I site, allowing antibody genes to be cloned as *Sfi*I-*Not*I fragments. A peptide tag (17, 18) was introduced directly after the *Not*I site by cloning an oligonucleotide cassette, and followed by an amber codon introduced by site-directed mutagenesis (19) (Figure 1D).

A range of constructs (see Figure 2) were made from a clone (essentially construct II in pUC19) designed for expression in bacteria of a soluble Fab fragment (13) from the mouse anti-phOx (2-phenyl-5-oxazolone) antibody NQ10.12.5 (20). In construct II, the V-regions are derived from NQ10.12.5 and attached to human Cκ and CH1 (γ1 isotype) constant domains: the C-terminal cysteine residues, which normally form a covalent link between light and heavy antibody chains, have been deleted from both the constant domains (M.Dreher and E.S.Ward unpublished). To clone heavy and light chain genes together as Fab fragments (construct II) or as separate chains (constructs III and IV) for phage display, DNA was amplified from construct II by PCR to introduce a *Not*I restriction site at the 3' end, and at the 5' end either an *Apa*LI site (for cloning into fd-tet-DOG1) or *Sfi*I site (for cloning into pHEN1). The primers FABNOTFOK with VH1BACKAPA (or VH1BACKSFI15) were used for PCR amplificatiion of genes encoding Fab fragments (construct II), the primers FABNOTFOH with VH1BACKAPA (or VH1BA-CKSFI15) for heavy chains (construct III), and the primers FABNOTFOK and MVKBAAPA (or MVKBASFI) for light chains (construct IV).

The single-chain Fv version of NQ10.12.5 (construct I) has the heavy (VH) and light chain (Vκ) variable domains joined by a flexible linker $(Gly_4Ser)_3$ (12) and was constructed from construct II by 'splicing by overlap extension' (21) as in Refs. 9 and 22. The assembled genes were reamplified with primers VK3F2NOT and VH1BACKAPA (or VH1BACKSFI15) to append restriction sites for cloning into fd-tet-DOG1 (*Apa*LI-*Not*I) or pHEN1 (*Sfi*I-*Not*I).

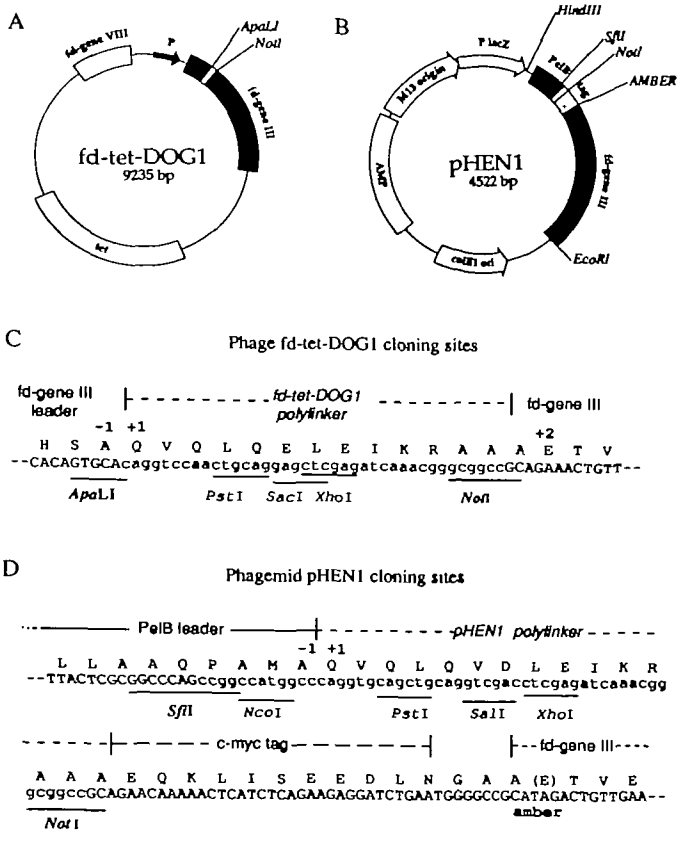

Figure 1. Phage and phagemid vectors used for display of antibody fragments on the surface of phage. (A) Structure of vector fd-tet-DOG1. (B) Structure of vector pHEN1. (C) Sequence of fd-tet-DOG1 cloning sites. (D) Sequence of pHEN1 cloning sites.

Table 1. Overview of phOx-BSA ELISA results of phage and phagemid constructions.

	Phage/Phagemid†	Helper phage	Binding to phOx*	Chain(s) displayed#	Chain as gene III fusion#	Soluble chain(s)#
A	fd-tet-DOG1		non binding	none		
	fd-tet-DOG1-I		binding	scFv	scFv	
	fd-tet-DOG1-II		binding	Fab	light chain	heavy chain
	pHEN1	VCSM13	non binding	none		
	pHEN1-I	VCSM13	binding	scFv	scFv	
	pHEN1-II	VCSM13	binding	Fab	light chain	heavy chain
B	pHEN1-I (HB2151)		binding			scFv§
	pHEN1-II (HB2151)		binding			Fab§
C	fd-tet-DOG1-III		non binding	heavy chain	heavy chain	
	fd-tet-DOG1-IV		non binding	light chain	light chain	
	pHEN1-III (HB2151)	VCSM13	non binding	none		heavy chain
	pHEN1-III (HB2151)	fd-tet-DOG1-IV	binding	Fab	light chain	heavy chain
	pHEN1-IV (HB2151)	VCSM13	non binding	none		light chain
	pHEN1-IV (HB2151)	fd-tet-DOG1-III	binding	Fab	heavy chain	light chain

*Phage were considered to be 'binding' if OD_{405} of sample was at least 10-fold greater than background in ELISA; †*E.coli* TG1 was used for the growth of the phage unless the use of *E.coli* HB2151 is specifically indicated; # Information deduced from genetic structure and in accordance with binding data; §Result confirmed experimentally by Western blot (for Fab, see Figure 3).

Rescue of phage and phagemid particles

Constructs I–IV (Figure 2) were introduced into both fd-tet-DOG1 and pHEN1. Phage fd-tet-DOG1 (and fd-tet-DOG1-I, II, III or IV) was taken from the supernatant of infected *E.coli* TG1 after shaking at 37°C overnight in 2×TY medium (23) with 12.5 μg/ml tetracycline, and used directly in ELISA. Phagemid pHEN1 (and pHEN1-I and II) in *E.coli* TG1 (*supE*) were grown overnight in 2 ml 2×TY medium, 100 μg/ml ampicillin, and 1% glucose (without glucose, expression of g3p prevents later superinfection by helper phage). 10 μl of the overnight culture was used to inoculate 2 ml of 2×TY medium, 100 μg/ml ampicillin, 1% glucose, and shaken at 37°C for 1 hour. The cells were washed and resuspended in 2×TY, 100 μg/ml ampicillin, and phagemid particles rescued by adding 2 μl (10^8 pfu) VCSM13 helper phage (Stratagene). After growth for one hour, 4 μl kanamycin (25 mg/ml) was added, and the culture grown overnight. The phagemid particles were concentrated 10-fold for ELISA by precipitation with polyethylene glycol (as in Ref. 7).

For assembly of heavy and light chains expressed from different vectors, phagemid (pHEN1-III or IV) was grown in *E.coli* HB2151 (a non-suppressor strain) to allow production of soluble chains, and rescued as above except using helper phage with partner chains as fusions to g3p (10^9 TU fd-tet-DOG1-IV or III respectively) and 2 μl tetracycline (12.5 mg/ml) in place of kanamycin.

Induction of soluble scFv and Fab

E.coli HB2151 was infected with pHEN phagemid (pHEN1-I or II), and plated on TYE (23), 100 μg/ml ampicillin plates. Colonies were shaken at 37°C in 2×TY medium, 100 μg/ml ampicillin, 1% glucose to OD_{550} = 0.5 to 1.0. Cells were pelleted, washed once in 2×TY medium, resuspended in medium with 100 μg/ml ampicillin, 1 mM isopropyl β-D-thiogalactoside (IPTG), and grown for a further 16 hours (18). Cells were pelleted and the supernatant, containing the secreted chains, used directly in ELISA.

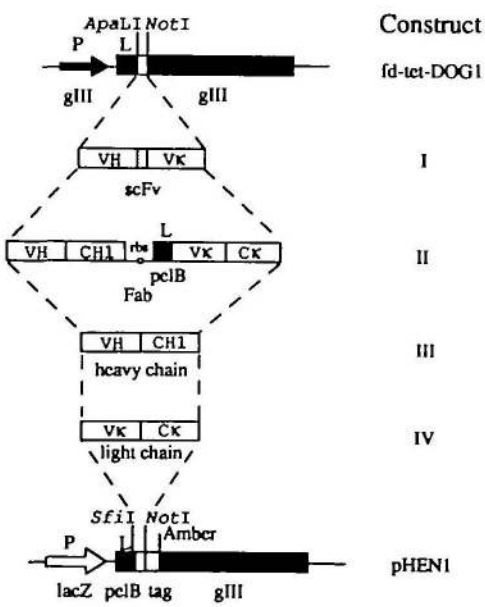

Figure 2. The antibody constructs cloned into fd-tet-DOG1 and pHEN1 for display on the surface of phage. Constructs I, II, III, and IV were cloned into both fd-tet-DOG1 (as *Apa*LI-*Not*I fragments) and pHEN1 (as *Sfi*I-*Not*I fragments). All the constructs contained the heavy chain (VH) and light chain (Vκ) variable regions of the mouse anti-phOx antibody NQ10.12.5. The constant domains were human Cκ and CH1 (γ1 isotype).

ELISA

Detection of phage binding to 2-phenyl-5-oxazolone (phOx) was performed as in Ref. 9: 96-well plates were coated with 10 μg/ml phOx-BSA or 10 μg/ml BSA in PBS overnight at room temperature, and blocked with PBS containing 2% skimmed milk powder. Phage(mid) supernatant (50 μl) mixed with 50 μl PBS containing 4% skimmed milk powder was added to the wells and assayed as described in Ref. 7. To detect binding of soluble scFv or Fab fragments secreted from pHEN1, the c-myc peptide tag was detected as in Refs. 17 and 18.

Western blot

Western blots were essentially as in Ref. 24. For detection of secreted proteins, 10 μl supernatant of induced cultures were subjected to SDS–PAGE (25) and proteins transferred by electroblotting to Immobilon-P (Millipore). Soluble heavy and light chain were, detected with goat polyclonal anti-human Fab antiserum (Sigma) and peroxidase conjugated rabbit anti-goat immunoglobulin (Sigma), each at a dilution of 1:1000. The tagged Vκ domain was detected with 9E10 antibody (1:1000) and peroxidase conjugated goat anti-mouse immunoglobulin (Fc specific) (1:1000) (Sigma) as in Ref. 18 or with a peroxidase labeled anti-human Cκ antiserum (Dako). 3,3′-diaminobenzidine (DAB; Sigma) was used as peroxidase substrate (26).

RESULTS

Vectors for display of scFv and Fab fragments on phage

We have used phage and phagemid vectors to display antibody fragments on the surface of filamentous phage. The phage vector, fd-tet-DOG1 (Ref. 9; Figure 1A and C) is based on the vector fd-tet (15) and has restriction sites (*Apa*LI and *Not*I) for cloning antibody (or other protein) genes for expression as fusions to the N-terminus of the phage coat protein g3p. Transcription of the antibody-g3p fusions in fd-tet-DOG1 is driven from the gene III promoter and the fusion protein targetted to the periplasm by

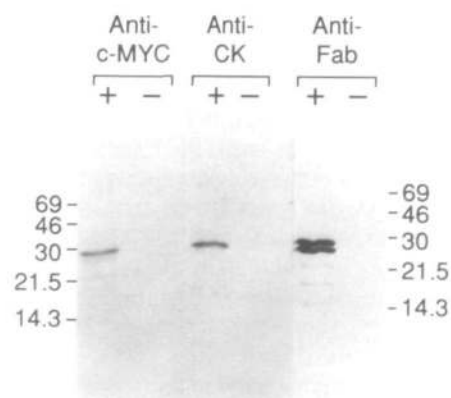

Figure 3. Western blot of supernatant taken from pHEN1-II (+) or pHEN1 (−) cultures in *E.coli* HB2151, showing secretion of Fab fragment from pHEN1-II only. The anti-human Fab detects both heavy and light chain. Due to the attached c-myc tag, the light chain, highlighted by both anti-c-myc tag and anti-human Cκ antisera, is slightly larger (calculated M_r 24625) than the heavy chain (calculated M_r 23145).

means of the g3p leader (Figure 1C). Fab and scFv fragments of NQ10.12.5 cloned into fd-tet-DOG1 for display were shown to bind to phOx-BSA (but not BSA) by ELISA (Table 1A).

The phagemid vector, pHEN1 (Figure 1B and D), is based on pUC119 (16) and contains restriction sites (*Sfi*I and *Not*I) for cloning the fusion proteins. Here the transcription of antibody-g3p fusions is driven from the inducible lacZ promoter and the fusion protein targetted to the periplasm by means of the pelB leader (13). Phagemid was rescued with VCSM13 helper phage in 2×TY medium containing no glucose or IPTG: under these conditions the natural leakiness of the promoter provided sufficient expression of antibody-g3p. Increased expression by IPTG induction led to cell death due to the toxicity of the fusion protein. Fab and scFv fragments of NQ10.12.5 cloned into pHEN1 for display were shown to bind to phOx-BSA (but not BSA) by ELISA (Table 1A).

The phagemid pHEN1 has the advantage over phage fd-tet-DOG1 in that antibody can be produced either for phage display (by growth in *sup*E strains of *E.coli*) or as a tagged soluble fragment (by growth in non-suppressor strains), as we introduced a peptide tag (17, 18) and amber codon between the antibody and g3p. Secretion of soluble Fab fragments from pHEN1-II or scFv fragments from pHEN1-I was demonstrated after growth in *E.coli* HB2151 and induction with IPTG using Western blots (Figure 3). With the scFv, the fragments were detected using the 9E10 anti-myc tag antibody (17, 18) (data not shown). With the Fab, only the light chain was detected by 9E10 (or anti-human Cκ) antibody, as expected, while the anti-human Fab antiserum detected both heavy and light chains. Binding of the soluble scFv and Fab fragments to phOx-BSA (but not to BSA) was also demonstrated by ELISA (Table 1B). Thus scFv and Fab fragments can be displayed or secreted as soluble fragments from the same phagemid vector.

Separate vectors to encode Fab heavy and light chains

The heavy and light chains of Fab fragments can be encoded together in the same vector (see above) or in different vectors. To demonstrate this we cloned the heavy chain (construct III) into pHEN1 (to provide soluble fragments) and the light chain (construct IV) into fd-tet-DOG1 (to make the fusion with g3p). The phagemid pHEN1-III, grown in *E.coli* HB2151 (non-suppressor) was rescued with fd-tet-DOG1-IV phage, and phage(mid) shown to bind to phOx-BSA, but not to BSA (Table 1C). This demonstrates that soluble heavy chain is correctly associating with the light chain anchored to the g3p, since neither heavy chain nor light chain alone bind antigen (Table 1C).

Similar results were obtained in the reverse experiment (with phagemid pHEN1-IV and fd-tet-DOG1-III phage) in which the light chain was produced as a soluble molecule and the heavy chain anchored to g3p (Table 1C). Hence a Fab fragment is assembled on the surface of phage by fusion of either heavy or light chain to g3p, provided the other chain is secreted using the same or another vector (Figure 4).

The resulting phage population is a mixture of fd phage and rescued phagemid. The ratio of the two types of particle was assessed by infecting log phase *E.coli* TG1 and plating on TYE plates with either 15 μg/ml tetracycline (to select for fd-tet-DOG1) or 100 μg/ml ampicillin (to select for pHEN1). The titre of fd-tet-DOG1 phage was 5×10^{11} TU/ml and the titre of pHEN1 2×10^{10} TU/ml, indicating a packaging ratio of 25 phage per phagemid.

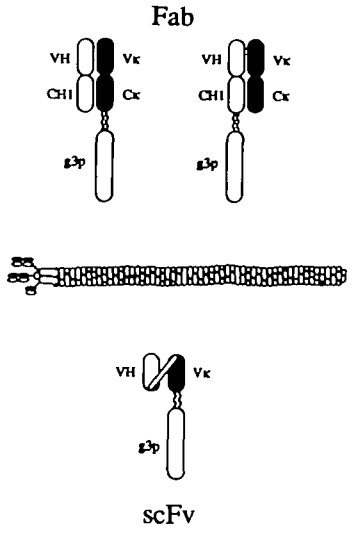

Figure 4. Three ways of displaying antibody fragments on the surface of phage by fusion to g3p.

DISCUSSION

The antigen-binding site of an antibody is formed by two domains, the heavy (VH) and light (VL) chain variable domains, which are on different polypeptide chains. These two variable domains can be expressed on the same polypeptide if they are joined artificially by a flexible linker (11, 12) to form single-chain Fv fragments (scFv). Previously we demonstrated that scFv antibody fragments can be displayed on the surface of fd phage by fusion to the amino terminus of g3p, that these 'phage antibodies' bind antigen (7), and that rare phage can be selected from large libraries (7, 9). However, scFv fragments often have affinities lower than the parent antibody (27).

Here we have demonstrated an alternative strategy involving display of the heterodimeric antibody Fab fragments on the surface of phage. One of the chains is fused to g3p and the other is secreted in soluble form into the periplasmic space of the *E.coli* where it associates non-covalently with the g3p fusion, and binds specifically to antigen. Either the light or heavy chain can be fused to the g3p: they are displayed on the phage as Fab fragments and bind antigen (Figure 4). We have described both phage and phagemid vectors for surface display: phagemids are probably superior to phage vectors for creation of large phage display libraries in view of their higher transfection efficiencies — two to three orders of magnitude higher, allowing larger libraries to be constructed. Our phagemid vector, pHEN1 also allows the expression of soluble Fab fragments in non-suppressor *E.coli*.

We have also demonstrated that heavy and light chains encoded on the same vector (construct II), or on different vectors (constructs III and IV) can be displayed as Fab fragments. This offers two distinct ways of making random combinatorial (28) libraries for display. Libraries of heavy and light chain genes, amplified by PCR, could be randomly linked by a 'PCR assembly' process (9, 22) based on 'splicing by overlap extension' (21), cloned into phage(mid) display vectors and expressed from the same promoter as part of the same transcript (construct II) as above, or indeed from different promoters as separate transcripts. Here the phage(mid) vector encodes and displays both chains. For a combinatorial library of 10^7 heavy chains and 10^7

light chains, the potential diversity of displayed Fab fragments (10^{14}) is limited by the transfection efficiency of bacterial cells by the vector (about 10^9 clones per μg cut and ligated plasmid at best) (29).

Alternatively, libraries of heavy and light chains could be cloned into different vectors for expression in the same cell, with a phage vector encoding the g3p fusion and a phagemid encoding the soluble chain. The phage acts as a helper, and the infected bacteria produce both packaged phage and phagemid. Each phage or phagemid displays both chains but encodes only one chain and thus only the genetic information for half of the antigen-binding site. However, the genes for both antibody chains can be recovered separately by plating on the selective medium, suggesting a means by which mutually complementary pairs of antigen binding heavy and light chain combinations could be selected from random combinatorial libraries. For example, a light chain repertoire on fd phage could be used to infect cells harbouring a library of soluble heavy chains on the phagemid. The affinity purified phagemid library could then be used to infect *E.coli*, rescued with the affinity purified phage library, and the new combinatorial library subjected to a round of selection. Thus, antibody heavy and light chain genes are reshuffled after each round of purification. Finally, after several rounds, infected bacteria could be plated and screened individually for antigen-binding phage. Such 'dual' combinatorial libraries are potentially more diverse than those encoded on a single vector: by combining separate libraries of 10^7 heavy chain and 10^7 light chain phage(mid)s, the diversity of displayed Fab fragments (potentially 10^{14}) is limited only by the number of bacteria (10^{12} per litre). More simply, the use of two vectors should also facilitate the construction of 'hierarchical' libraries, in which a fixed heavy or light chain is paired with a library of partners (9), offering a means of 'fine-tuning' antibody affinity and specificity.

In conclusion the display of heterodimeric Fab fragments on the surface of phage offers new possibilities for making antibodies from diverse libraries of genes, and our strategy may serve as a model for the display of other multichain polypeptides.

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