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Ex12 helper phage improves the quality of a phage-displayed antibody library by ameliorating the adverse effect of clonal variations

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The quality of a phage-displayed antibody library deteriorates with clonal variations, which are caused by differentially expressed *Escherichia coli* antibody genes. Using the human Fab SP114 against the pyruvate dehydrogenase complex-E2 (PDC-E2), we created four *E. coli* TOP10F' clones with a pCMTG phagemid encoding Fab-pIII (pCMTG-Fab), Fd (V_H+C_{H1})-pIII (pCMTG-Fd), or light chain (L) (pCMTG-L), or the vector only (pCMTG-ΔFab) to investigate the effect of clonal variations in a defined manner. Compared to the others, the *E. coli* clone with pCMTG-Fab was growth retarded in liquid culture, but efficiently produced phage progenies by Ex12 helper phage superinfection. Our results suggest that an antibody library must be cultured for a short duration before helper phage superinfection, and that the Ex12 helper phage helped to alleviate the detrimental effect of clonal variation, at least in part, by preferentially increasing functional phage antibodies during phage amplification. [BMB reports 2011; 44(4): 244-249]

INTRODUCTION

Generating human antibodies with desired antigen-binding specificities from a human naïve immunoglobulin (Ig) repertoire *via* phage display technology is therapeutically valuable because the antibodies may induce less immunogenicity in humans. However, such a phage display is difficult to achieve due to technical complications, including vector designs, bacterial strains, culture conditions and, most importantly, clonal variations caused by incorporating PCR errors into antibody genes, low efficiency of translating certain codons of eukaryotic antibody genes, and degradation or aggregation of antibody-pIII fusion proteins (1-4). Indeed, most phagemid vectors utilized in constructing an antibody display library use the lac promoter to control the expression of scFv-pIII or Fab-pIII fusion proteins. The fusion proteins are produced in cells without IPTG induction due to the natural leakiness of the promoter (5, 6), which is enough to result in a strong selection against plasmid maintenance and integrity (7). Therefore, clonal variations can affect the quality of an antibody library by several mechanisms, including negative selection of the cells expressing scFv-pIII fusions in an antibody library, differential stability of antibody-pIII fusions on a recombinant phage, and biased amplification of non-functional progeny phages in the final phage antibody repertoire (8, 9).

Despite numerous successful demonstrations of phage display technology for antibody generation (10), the technological limitation caused by clonal variations is still problematic to reliably isolated target-specific antibodies, particularly from a human naïve Ig repertoire (2, 9-11). In this study we attempted to analyze the deleterious effects of clonal variation using *E. coli* clones with pCMTG phagemid constructs encoding Fab-pIII (pCMTG-Fab), Fd (V_H+C_{H1})-pIII (pCMTG-Fd), light chain (L) ($V_L+C_{Lκ}$) (pCMTG-L), or the vector only (pCMTG-ΔFab) using a Fab SP114 that is specific for pyruvate dehydrogenase complex-E2 (PDC-E2) as a model system. We demonstrated that the deleterious effects of clonal variations can be amended partly by using the Ex12 helper phage, a mutant M13K07 helper with two amber codons at the gIII (gIII-amber) (12) *via* the preferential production of progeny phages from the *E. coli* clones that are capable of producing functional antibody fragments.

RESULTS AND DISCUSSION

Clonal variations in *E. coli* cells expressing antibody genes from a naïve Ig repertoire

A library size considerably greater than 5×10^8 is desirable to identify antibodies with desired affinities or catalytic activities

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(13). The antibody repertoire should be more than 10^{10} to obtain high-affinity antibodies (10^{-9}-10^{-10} M) (14). Unfortunately, recombinant phages obtained from an antibody library may not truly represent the antibody repertoire of the library, because *E. coli* clones can produce biased yields of functional recombinant phages due to differential translation or stability of the antibody-pIII fusions produced by *E. coli* (15). Because a human naïve Ig repertoire antibody library would have great value for developing therapeutic antibody leads, we decided to analyze clonal variations in the *E. coli* clones obtained from a human naïve Fab antibody library constructed with the pCMTG phagemid vector from the IGT-hu library (unpublished, IG Therapy Co.). Twenty-four *E. coli* clones that contained the pCMTG phagemid and that both had Fd and L gene inserts were chosen, and an ELISA was performed to determine the expression of soluble Fd-pIII fusions and kappa L chain fragments by *E. coli* clones (Fig. 1). The results showed drastic clonal variations in the expression of soluble Fd-pIII and kappa L chain fragments among the *E. coli* clones. For example, the #6, #8, #12, and #19 clones expressed a high level of Fd-ΔpIII fusions, and the #2, #14, #15, and #16 clones produced a high level of L chain fragments. The #7 and #10 clones produced no or only a trace amount of Fd-ΔpIII and L chain fragments, indicating that the naïve origin Fd and kappa L chains are translated at different rates in *E. coli*.

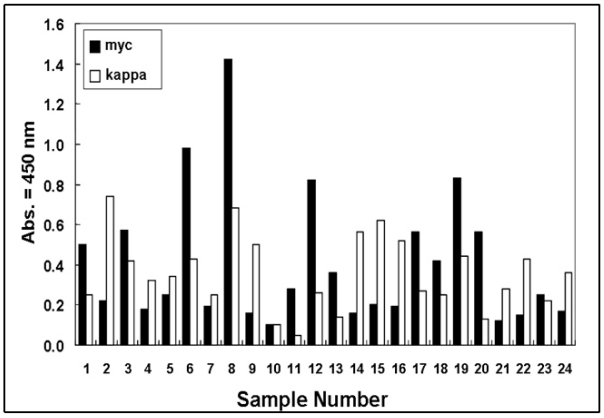

Fig. 1. Determination of soluble Fd-pIII or L chain fragments produced by *E. coli* clones obtained from the human naïve Fab library IGT-hu. Twenty-four *E. coli* colonies were randomly chosen from the human naïve Fab library IGT-hu and grown in 2× YT/A medium supplemented with 0.1 mM IPTG overnight to prepare the culture supernatant. Microtiter plates were coated with *E. coli* culture supernatant, and an ELISA was performed to determine the presence of soluble Fd-pIII or kappa L chain fragments in the culture supernatant using mouse anti-myc mAb followed by a HRPO conjugated secondary goat anti-mouse IgG pAb or by HRPO conjugated goat anti-human kappa L chain pAb, respectively. The signal was visualized with TMB substrate and analyzed by measuring OD_{450nm}. Data are shown as average of two experiments (SD).

Creating *E. coli* clones that show the defined antibody fragment expression patterns and their growth kinetics in liquid culture

To analyze the effect of *E. coli* clonal variations more definitively, pCMTG-Fab, pCMTG-Fd, pCMTG-L, and pCMTG-ΔFab vectors were constructed using the Fd and L chain gene fragments of SP112, an antigen-binding fragment (Fab) clone specific for PDC-E2 as a model system, and electroporated it into suppressive *E. coli* TOP10F' cells (Fig. 2A). The pCMTG vector contains the myc tag sequence and the truncated gIII fragment encoding aa 214-406 residues of pIII downstream of the cloning sites to facilitate detection of Fd-pIII fusion molecules with an anti-myc tag mAb.

Monoclonal or polyclonal *E. coli* clones containing the pCMTG-Fab, pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab phagemids were grown in 2× YT/A or in 2× YT/AG liquid medium at 27°C for 16 h, and growth kinetics were determined by measuring the medium at OD_{600nm} every 2 hours (Fig. 2B). The initial cell concentration was approximately 2×10^7 cells/ml (OD_{600} = 0.02), and a suboptimal temperature was used to grow the cells to facilitate production of functional Fab fragments (16). The growth kinetics of the monoclonal *E. coli* cultures showed that the *E. coli* clones containing the pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab clones had similar growth rates, suggesting that expression of the Fd-ΔpIII fusion or the L chain fragment did not affect growth of *E. coli* (Fig. 2Ba and b). However, the *E. coli* clone containing pCMTG-Fab showed substantial growth retardation compared to the other three *E. coli* clones in 2× YT/A medium (Fig. 2Ba), and supplementing with 2% glucose improved the growth of the clone, although not substantially, indicating that suppressing the *lac* promoter with glucose is insufficinet to restore the normal growth of *E. coli* that express functional Fab-pIII fusion molecules (Fig. 2Bb). Next, we mixed four monoclonal *E. coli* clones at different ratios (pCMTG-Fab : pCMTG-Fd : pCMTG-L : pCMTG-ΔFab = 25 : 25 : 25 : 25, 50 : 16.7 : 16.7 : 16.7 or 75 : 8.3 : 8.3 : 8.3%), and the growth kinetics of the polyclonal *E. coli* culture were measured as described above. As expected, the polyclonal *E. coli* culture containing a higher percentage of the *E. coli* clone with pCMTG-Fab showed a slower growth rate (Fig. 2Bc and d) and supplementing with 2% glucose was only slightly beneficial, as in Fig. 2Bb.

Small aliquots of the cell suspension were obtained from the cells used for Fig. 2Bc and spread onto 2× YT/AG plates. The presence of the *E. coli* clone with pCMTG-Fab in the polyclonal cell cultures at 0, 4 , 8 , 12, and 16 h after incubation was analyzed by ELISA using microtiter plates coated with rPDC-E2 (data not shown). The results revealed that the percentage of *E. coli* clones with pCMTG-Fab dropped drastically even after 4 h of liquid culture, and almost no *E. coli* clone containing pCMTG-Fab was detected at 12 or 16 h if the culture contained less than 50% of the *E. coli* clone with pCMTG-Fab at the initial culture. This rapid reduction in the *E. coli* clone with pCMTG-Fab did not seem to be caused by deleting

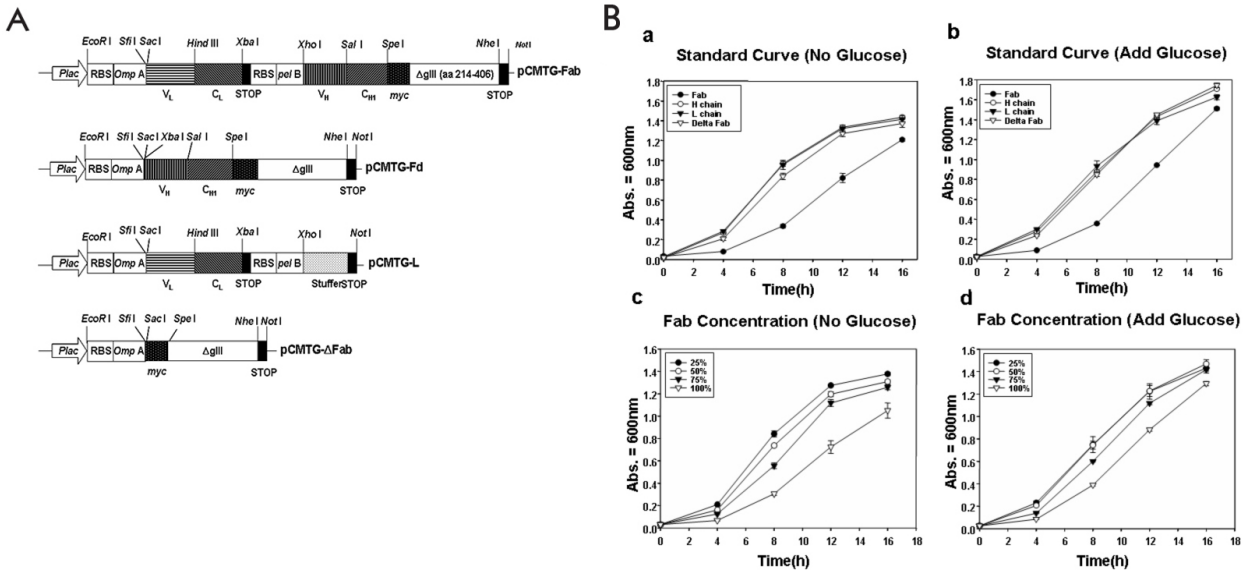

Fig. 2. Scheme of the pCMTG phagemid vector and growth kinetics of the *E. coli* liquid culture. (A) Diagram of the pCMTG phagemid vector constructs used in this study. (B) The *E. coli* clones carrying pCMTG-Fab, pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab at the monoclonal level (a and b), or polyclonal mixtures of four *E. coli* clones containing 25, 50, 75, or 100% of the *E. coli* clones carrying pCMTG-Fab (c and d) were grown separately in 2× YT/A (a and c) or 2× YT/AG medium (b and d). The cultures were grown at 27°C in a shaking incubator, and 1 ml aliquots were removed from the cultures at 0, 4, 8, 12 or 16 h after incubation. The samples were then measured at OD_{600} using a spectrophotometer. The experiments were conducted in triplicate, and the data are shown as average ± standard deviation (SD).

Table 1. Phage titer obtained from the monoclonal *E. coli* clones by helper phage superinfection at different time points

Helper phage	Time for phage amplification (h)*	Phage titer from the monoclonal *E. coli* clones (phage/ml)			
		pCMTG-Fab	pCMTG-Fd	pCMTG-L	pCMTG-ΔFab
M13KO7	0	N/D †	N/D	N/D	N/D
	4	6.0×10^7	6.0×10^7	1.5×10^8	2.0×10^8
	8	1.7×10^8	1.4×10^8	3.0×10^8	4.5×10^8
	12	6.0×10^8	1.8×10^8	6.0×10^8	9.0×10^8
	16	7.5×10^8	2.0×10^8	9.0×10^8	9.0×10^8
Ex12	0	N/D	N/D	N/D	N/D
	4	1.4×10^7	N/D	N/D	N/D
	8	2.3×10^7	N/D	N/D	N/D
	12	4.5×10^7	N/D	N/D	N/D
	16	2.7×10^7	N/D	N/D	N/D

*Time (h) was measured from when cells were resuspended with 10× volume of fresh 2× YT/amp/kan just after 1 h infection with either M13KO7 or Ex12 helper phage at 37°C, followed by brief centrifugation, †Not detectable by phage ELISA ($< 1.6 \times 10^5$ phage/ml)

the Fd or L chain genes from the pCMTG-Fab vector because all 24 *E. coli* colonies detected at 16 h of incubation with 100% pCMTG-Fab produced functional Fab-pIII molecules. Clonal variations severely deteriorate the quality of an antibody library due to outgrowth of *E. coli* subpopulations that fail to express functional Fab-ΔpIII molecules frequently present in antibody libraries under normal conditions in liquid medium (17). However, the exact clonal variation parameters have not been clearly elcuidated using the experimental setups so far. Our results provide a very critical parameter for constructing an antibody library for the first time in that any Fab antibody library, regardless of its naïve or synthetic Ig repertoire, must contain more than 75% of the *E. coli* populations that produce both heavy and light chain fragments.

Analysis of recombinant phage progenies amplified from the *E. coli* clones using the M13KO7 or Ex12 helper phages

Recombinant phage progenies were prepared from the *E. coli*

TOP10'F clones with pCMTG-Fab, pCMTG-Fd, the pCMTG-L, or the pCMTG-ΔFab by phage rescue using the M13KO7 or Ex12 helper phages. The Ex12 helper phage is a mutant M13K07 helper with two UAG amber codons at the gIII (gIII-amber) (12), and, therefore, it does not produce wild type pIII in *E. coli* host strains belonging to the supE genotype such as TOP10'F cells. The recombinant phage progeny titer was determined by a phage ELISA and is presented in Table 1. During phage amplification using the M13KO7 helper phage, the *E. coli* clones with pCMTG-Fab, pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab yielded comparable phage titers, implying that approximately 75% of phage progenies may not display functional antibody fragments in this phage preparation. However, phage progenies were produced only from the *E. coli* clone with pCMTG-Fab but not from the cells with pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab when the Ex12 helper phage was used for phage amplification. The presence of excessive non-functional phage progenies decreases the chances of isolating specific binders from an antibody library by lowering panning efficiency (18, 19). Therefore, it is reasonable to state that the Ex12 helper phage increases the quality of a phage-displayed antibody library by reducing amplification of non-functional phage progenies, thereby facilitating isolation of specific binders from an antibody library.

The observation that the *E. coli* clones carrying pCMTG-L or pCMTG-ΔFab were not able to produce phage progenies using Ex12 helper phage superinfection was expected because of the existence of two amber codons in the gIII of Ex12 helper phage, which prevent the production of a functional wild type pIII in the non-suppressive *E. coli* TOP10F' strain (20). Notably, the *E. coli* clone with pCMTG-Fd also did not produce phage progenies, even though the cells expressed the same level of the Fd-ΔpIII fusion as the *E. coli* clone carrying pCMTG-Fab (data not shown). The reason would be that the Fd-ΔpIII fusion without the L chain fragment aggregated very easily by exposing the hydrophobic residues, resulting in the inefficient production of phage progenies.

Use of the Ex12 helper phage seemed to be additionally beneficial for avoiding outgrowth of the *E. coli* clones that fail to express functional antibody fragments, because a phage amplification as short as 4 h was enough to obtain a sufficient phage antibody titer (Table 1). To confirm this finding, a phage ELISA was performed to determine the PDC-E2-binding intensity of the recombinant phage that had been obtained using the M13KO7 or Ex12 helper phages. The results showed that phage antibodies produced using the Ex12 helper phage exhibited almost identical PDC-E2-specific binding signals regardless of the phage amplification time and produced stronger binding signals than those produced using the M13KO7 helper phage despite the fact that 3- to 7-fold less phage was assayed by ELISA (Fig. 3).

We clearly showed that the outgrowth of *E. coli* subpopulations not expressing functional Fab fragments caused overproduction of non-functional phage progenies and decreased

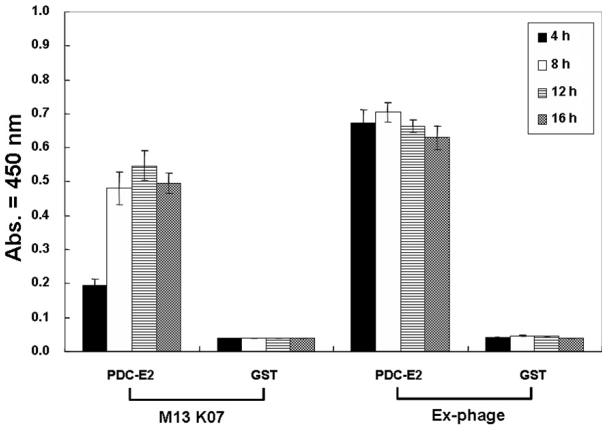

Fig. 3. Phage ELISA showing antigen-binding specificity of the phage antibodies obtained from the *E. coli* clone with pCMTG-Fab using M13KO7 or Ex12 helper phages. Recombinant phages were obtained from the *E. coli* carrying pCMTG-Fab by phage rescue using the M13KO7 or Ex12 helper phages after 4, 8, 12 or 16 h of phage amplification, and the phage ELISA was performed using a 96-well ELISA plate coated with rPDC-E2 or GST (negative control). Bound phages were detected with HRPO conjugated goat anti-M13 polyclonal antibody. The signal was visualized with TMB substrate, and analyzed by measuring the samples at OD_{450nm}. The experiments were conducted in triplicate, and data are shown as the average ± standard deviation (SD).

library quality. Use of the Ex12 helper phage ameliorated this deleterious effect of *E. coli* clonal variations, at least in part, through the preferential production of phage progenies from the *E. coli* clone that expressed functional antibody fragments. The chance of isolating target-specific phage antibodies depends on the panning efficiency, and the use of the Ex-12 helper phage would be quite beneficial to isolate specific binders from a phage-displayed antibody library by a drastic increase in the proportion of antibody-displaying phage progenies over non-functional phages in a recombinant phage preparation.

MATERIALS AND METHODS

Construction of recombinant vectors

The human Fd and L chain genes of the Fab SP112 specific for PDC-E2 (21) were PCR amplified from the SP112 using human-specific Ig primers in the ImmunoZap kit (Stratagene, La Jolla, CA, USA) and Ex-*Taq* polymerase (Takara, Shiga, Japan) and cloned into the pCMTG phagemid vector to construct pCMTG-Fab (IG Therapy Co., Seoul, South Korea, unpublished) according to standard cloning procedures (22). To generate pCMTG-Fd, the gene fragment containing Fd + myc tag + gIII region was obtained from pCMTG-Fab by PCR amplification using the following primers (sense 5'-GGGGAGCTCCTCGAGGTGCAGCT(G/A)CAG(G/C)AGT-3' and anti-sense 5'-AGCA TCACTAGTACAAGATTTGGGCTC-3') (Bioneer Co., Seoul, South

Korea). The amplified product was digested with *Sac*I/*Spe*I and cloned into pCMTG. To construct pCMTG-L, the Fd gene fragment of pCMTG-Fab was replaced with a 300 bp DNA stuffer fragment using *Xho*I/*Spe*I restriction enzymes. The resulting vector was treated again with *Spe*I/*Nhe*I to remove the gIII fragments, self-ligated, and electroporated into amber-non-suppressive *E. coli* TOP10F' cells. To create pCMTG-ΔFab, which does not encode the L chain fragment or Fd-pIII fusion, pCMTG-Fab was treated with *Sac*I/*Spe*I, and ~3.2 kb of the vector moiety was recovered from an agarose gel and ligated with an *Sac*I-*Sma*I-*Eco*RV-*Spe*I adaptor that had been generated by annealing two oligonucleotides (5'-CACCCCGGGATATCT A-3', and 5'-CTAGTAGATATCCCCGGGTGAGCT-3'), followed by electroporation into TOP10F' cells.

Liquid culture of the *E. coli* clones

E. coli TOP10F' cells housing pCMTG-Fab, pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab were spread onto 2× YT agar plates containing 50 μl/ml ampicillin and 2% glucose (2× YT/AG plate) and grown in a 27°C incubator overnight. Cells were harvested by scraping the *E. coli* colonies with a sterile glass rod in the presence of 5 ml 2× YT/AG medium, and the cell concentration was adjusted to OD_{600} = 1.0. For the growth kinetic studies, each of these *E. coli* suspensions was diluted with 20× volumes of pre-warmed fresh 2× YT/A or 2× YT/AG medium and incubated in a shaking incubator at 27°C. Absorbance of the cultures at 600 nm was measured every 4 h, and small aliquots were spread onto 2× YT/AG plates. To determine the effect of clonal variations in liquid culture, the *E. coli* clone with pCMTG-Fab was diluted with a mixture of the *E. coli* TOP10F' clones carrying pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab at the same ratio (1 : 1 : 1), so that percentage of the *E. coli* clone with pCMTG-Fab were 25, 50, 75, or 100%. Then, the polyclonal *E. coli* mixtures were diluted with 20× volumes of fresh 2× YT/A or 2× YT/AG medium and incubated in a shaking incubator at 27°C. During the incubation, absorbance of the cultures at 600 nm was measured every 4 hours, and small aliquots were spread onto 2× YT/AG plates as described above.

Enzyme-linked immunosorbent assay (ELISA)

A recombinant inner lipoyl domain of human PDC-E2 (residues 91-227) fused to glutathione S-transferase (GST) was prepared as described previously (23). *E. coli* culture supernatant containing soluble antibody fragments was obtained by growing *E. coli* colonies in sterile 96-well plates (Nunc, Roskilde, Denmark) containing 100 μl of 2× YT/A medium supplemented with 0.15 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) at 27°C for 16 h, and a standard ELISA was performed using Maxisorp 96-well plates (Nunc) coated with 50 μl of 10 μg/ml recombinant PDC-E2 (rPDC-E2) or GST in a coating buffer (0.1 M $NaHCO_3$, pH 9.6). A mouse anti-myc tag monoclonal antibody followed by horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG secondary antibody (Sigma) or by a HRPO conjugated goat anti-human kappa light chain (Sigma) was used to detect the Fd-pIII fusion or the L chain molecule, respectively. The binding signals were visualized by using 3.3',5.5'-tetramethyl benzidine substrate (Sigma), followed by adding equal volumes of 1 N HCl into each well. Absorbance at 450 nm was measured using an ELISA reader (Biorad, Hercules, CA, USA).

Amplification of phage progenies and the phage ELISA

Recombinant phages were obtained from the *E. coli* TOP10F' cells with pCMTG-Fab, pCMTG-Fd, pCMTG-L, or pCMTG-ΔFab by phage rescue using M13KO7 (Amersham Pharmacia, Upsala, Sweden) or the Ex12 helper phage, according to standard protocols (12, 24). Small aliquots were obtained from the cultures at 0, 4, 8, 12, and 16 h after phage rescue, and the phage ELISA was performed to determine phage titer and binding reactivity of phage antibodies phage as described previously (12).

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