

Mutational analysis of the major coat protein of M13 identifies residues that control protein display

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Abstract

We have reported variants of the M13 bacteriophage major coat protein (P8) that enable high copy display of monomeric and oligomeric proteins, such as human growth hormone and streptavidin, on the surface of phage particles (Sidhu SS, Weiss GA, Wells JA. 2000. High copy display of large proteins on phage for functional selections. *J Mol Biol* 296:487–495). Here, we explore how an optimized P8 variant (opti-P8) could evolve the ability to efficiently display a protein fused to its N-terminus. Reversion of individual opti-P8 residues back to the wild-type P8 residue identifies a limited set of hydrophobic residues responsible for the high copy protein display. These hydrophobic amino acids bracket a conserved hydrophobic face on the P8 alpha helix thought to be in contact with the phage coat. Mutations additively combine to promote high copy protein display, which was further enhanced by optimization of the linker between the phage coat and the fusion protein. These data are consistent with a model in which protein display-enhancing mutations allow for better packing of the fusion protein into the phage coat. The high tolerance for phage coat protein mutations observed here suggests that filamentous phage coat proteins could readily evolve new capabilities.

Keywords: M13; mutagenesis; phage display; protein engineering

Phage display, the fusion of polypeptides to the surface of phage particles (Smith, 1985), expedites and simplifies the process of protein engineering (Clackson & Wells, 1994). A fusion protein-encoding plasmid enables convenient mutagenesis of the protein fusion to create large libraries of displayed proteins. Proteins with desired functions can be isolated by sorting or biopanning, and the protein sequence can be readily determined through sequencing of the encapsulated DNA. Amplification of selected phage particles in an *Escherichia coli* host facilitates multiple rounds of sorting (reviewed by Smith & Petrenko, 1997).

In a phagemid display system, the phagemid DNA contains an open reading frame encoding the display protein fused, through

a linker, to a copy of either the major coat protein (protein-8, P8) or the gene-3 minor coat protein (protein-3, P3). In theory, the choice of the phagemid coat protein should affect the protein display levels because P8 is present in ~2,700 per phage, whereas P3 is present in about five copies per phage. However, in practice protein display levels on P8 are strongly dependent on protein sequence and length, with longer polypeptides associated with decreased display (Kang et al., 1991; Iannolo et al., 1995; Kretzschmar & Geiser, 1995; Malik et al., 1996; Chappel et al., 1998).

Traditionally, the P3 and P8 proteins used in phagemids to anchor displayed proteins are wild-type phage proteins, which are optimized for functions unrelated to display of protein libraries. For example, P8 performs the dual roles of packaging the phage single stranded DNA and forming phage coat. For these functions, P8 assumes an extended alpha helix that packs around the DNA core in a right-handed coiled coil (Welsh et al., 1998). However, we have previously shown that optimized P8 variants (opti-P8s) can be selected for enhanced protein display (Sidhu et al., 2000b). Here, we explore the evolution of the P8 coat protein to embrace a new function, high-copy display of proteins.

Assembly and evolution of filamentous bacteriophage coat proteins are interesting both theoretically and practically. The TcpA coat protein of filamentous bacteriophage VPIÖ, for example, confers pathogenicity upon its host strain *Vibrio cholerae* (Karoulis et al., 1999). We examine how a single copy of P8, surrounded by

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; hGH, human growth hormone; IPTG, isopropylthio β -D-galactoside; opti-P8, optimized P8 variant; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); P3, protein-3, the gene-3 minor coat protein of M13 phage; P8, protein-8, the gene-8 major coat protein of M13 phage; SAV, streptavidin; Tween-20, polyoxyethylene sorbitan monolaurate; wt, wild-type. Amino acids are indicated by single or triple letter code. Mutations are designated by the single letter code for the starting amino acid, followed by its position in the amino acid sequence and the mutant amino acid.

wild-type (wt) P8 molecules, responds to mutations and selection for enhanced protein display. A single P8 molecule can tolerate a large number of mutations. Mutational analysis identifies the linker plus a limited patch of hydrophobic substitutions and conserved residues, which could interact with the phage core, as being primarily responsible for the enhanced functionality of high copy protein display.

Results

Mutagenesis of enhanced display P8 variants

To pinpoint residues most critical for high protein display by the protein display-optimized P8 variants, we made site-directed mutations that reverted individual residues of the opti-P8 molecules back to the corresponding wt P8 residue. Three opti-P8 selectants (1a, 2a, and 3a), each covering different 10 residue stretches of P8 (Fig. 1), were mutated back to wild-type, and levels of human growth hormone (hGH) display were assayed. Of the 25 back mutations, only three had large effects on the display of hGH (Fig. 2). F6P, I17S, and L25A reduced hGH display by greater than 10-fold. D1A, S3G, N20E *increased* protein display slightly (less than twofold), while the K2E, E12N, and A13S mutations reduced hGH display by approximately fourfold.

None of these point mutations individually account for the dramatic gains (50- to 100-fold) in protein display levels observed for fusion to the opti-P8 selectants (Sidhu et al., 2000b). To explore how combinations of mutations can increase protein display levels, two back mutations (E12N and A13S) with moderate effects on hGH display levels were combined with I17S, which had a large effect on hGH display levels (Fig. 2D). Combination of the back mutations decreased protein display in a predictable, additive fashion, suggesting that each mutation increases protein display levels noncooperatively.

Combination of mutants from different zones

As an extreme test of the tolerance for mutation in a phagemid P8 system, different P8 selectants (1a, 2a, and 3a; Fig. 1) were combined to produce hypermutated P8 variants. Two outcomes were envisioned for the grafting of mutations that were selected in separate experiments. Either the combined mutations would result in improved protein display levels, or the large number of mutations would drastically impair protein display levels. All combinations improved hGH display over levels accessible with fusion to wt P8, but not significantly beyond levels obtained with the original selectants (Fig. 3). 1a+2a+3a, a construct combining mutations from selectants 1a, 2a, and 3a, is particularly noteworthy. With an overall sequence 50% mutated from wild-type, 1a+2a+3a conserves only 5 of the first 30 residues of P8, but four of these conserved residues were generally selected as wild-type during protein display optimization (Fig. 1).

Optimization of the linker between phagemid P8 and the fusion protein

As mutations to the N-terminal half of P8 enhance display, the linker between the P8 N-terminus and the fusion protein could affect protein display levels. To test this hypothesis, a library of

A									
	1	2	3	4	5	6	7	8	9
wt	A	E	G	D	D	F	A	K	A
1a	D	K	S	E	K	F	A	R	D
1b	I	K	D	E	Q	F	A	R	-
1c	I	Y	I	K	E	F	A	-	N
1d	N	Y	V	-	Q	F	A	-	N
1e	-	K	A	E	E	F	A	E	-
1f	-	D	I	-	-	F	A	R	S

B										
	11	12	13	14	15	16	17	18	19	20
wt	F	N	S	-	Q	A	S	-	T	E
2a	Y	E	A	-	E	D	I	-	-	N
2b	Y	E	A	-	E	D	I	-	-	N
2c	Y	E	A	-	E	D	I	-	-	N
2d	Y	D	V	-	-	I	A	-	I	N
2e*	L	K	D	-	K	-	T	-	I	Q
2f*	Y	E	T	-	K	D	D	-	V	K
2g*	L	Q	N	-	H	S	A	-	S	K
2h*	Y	K	T	-	-	G	A	-	A	K
2i*	Y	K	T	-	K	S	I	-	N	K
2j*	Y	Q	-	-	-	I	I	-	A	Q
2k*	-	Q	-	-	K	D	T	-	D	-
2m*	-	E	N	-	-	-	T	-	-	K
2n†	-	D	A	-	K	D	I	-	S	Q
2p†	Y	E	T	-	E	D	A	-	S	-
2q†	Y	E	A	-	Y	-	A	-	-	K
2r†	-	E	A	-	-	G	V	-	V	K

C										
	21	22	23	24	25	26	27	28	29	30
wt	Y	I	G	Y	A	W	A	M	V	V
3a	L	F	F	L	L	G	T	V	H	L
3b	-	Y	L	N	I	L	-	V	Y	-
3c	F	-	R	V	T	-	T	-	Y	-
3d	V	-	R	-	V	M	S	-	Y	-
3e†	V	Y	S	H	I	L	T	-	I	F
3f†	V	V	V	F	V	-	V	L	H	N
3g†	I	V	-	H	L	L	-	V	Y	F
3h†	F	-	R	H	S	L	-	W	N	-

Fig. 1. Amino acid sequences of opti-P8s selected for increased hGH or *SAV display. Selectants were isolated from P8 libraries with mutations in (A) residues 1 through 10, (B) residues 11 through 20, or (C) residues 21 through 30. †Selectants obtained in this study from libraries fused to hGH with an optimized linker sequence (L1; Fig. 4). All other selectants were reported previously (Sidhu et al., 2000b). Hyphens denote wt sequences. The mutated region of each selectant is shown, with the rest of each sequence being identical to wt P8. Dark gray shading highlights positions that were mostly conserved as wild-type. Light gray shading indicates positions where non-wt side chains greatly increased protein display levels (Fig. 2).

14-residue linkers was used to replace the 10-residue Gly-Ser rich linker between hGH and wt P8. Each position was randomized with a set of nine, mostly hydrophilic amino acids (Ala, Arg, Asn, Asp, Gly, His, Pro, Ser, or Thr). Selection for maximized hGH display yielded many diverse sequences (Fig. 4). However, most of the selected linkers increased hGH display relative to the original linker (Fig. 5).

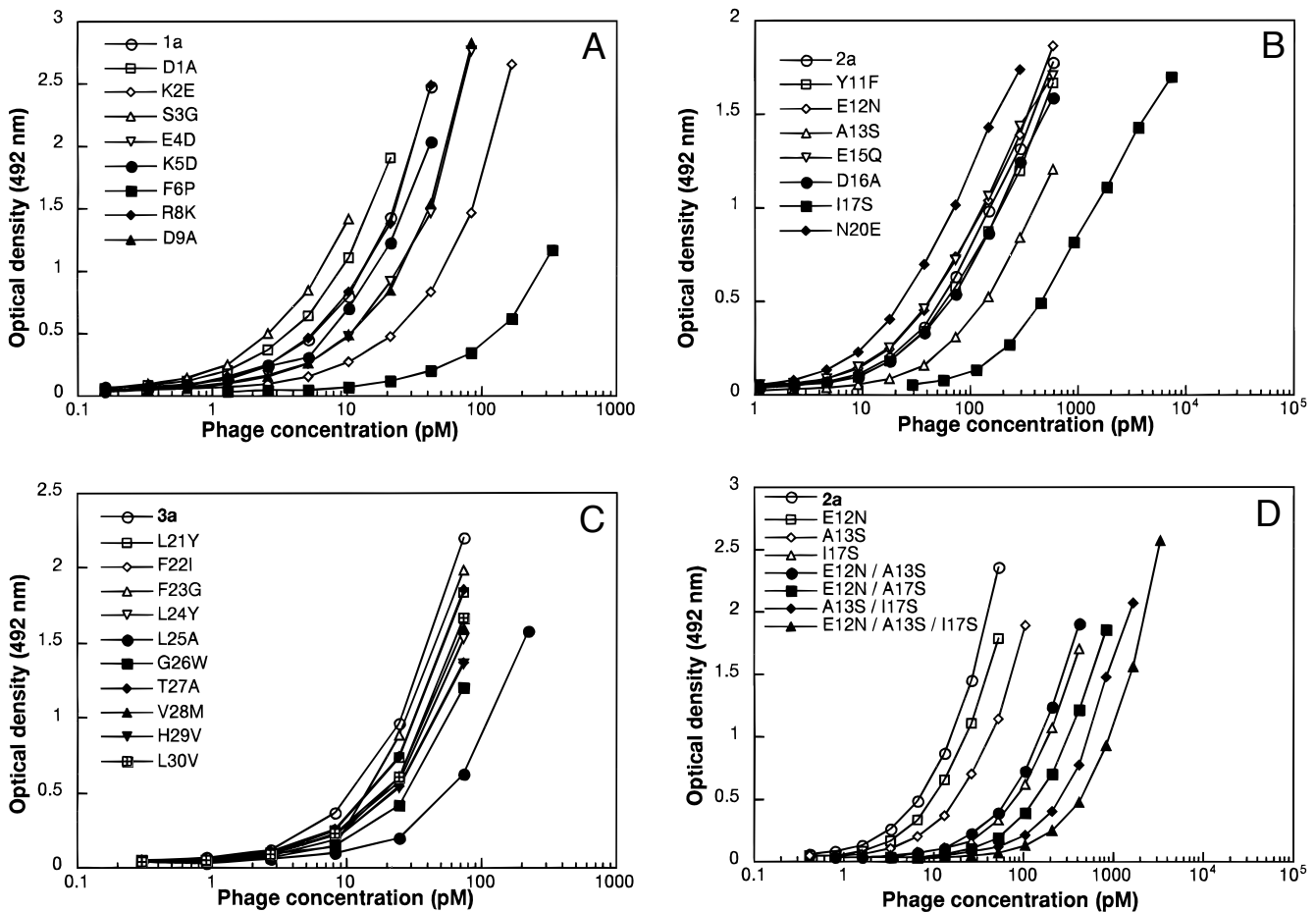


Fig. 2. Phage ELISAs of opti-P8 mutations back to wt P8 highlight key positions for enhanced display. **A:** hGH was fused to opti-P8 1a, or P8 variants derived from 1a with the labeled point mutations back to wild-type. In this selectant, the side chain at position 6 had the greatest effect on hGH display. **B:** Point mutations back to wild-type reveal that increased hGH display with opti-P8 2a is primarily due to a hydrophobic residue in position 17. **C:** Back mutations of opti-P8 3a identify Leu25 as the single residue most responsible for enhanced hGH display with this selectant. **D:** Combinations of several back mutations in opti-P8 2a demonstrate the additive effects of protein display enhancing mutations. Constructs were grown using identical conditions. Each construct shown here is from a single assay plate, developed using a phage ELISA with an anti-hGH monoclonal antibody as the capture target. All assays were repeated in triplicate.

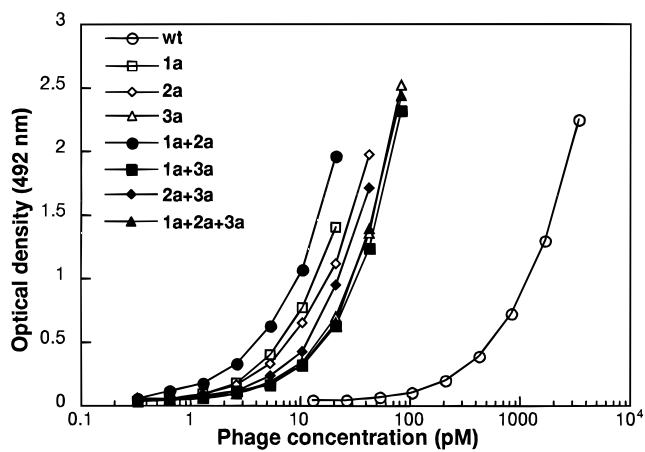


Fig. 3. Phage ELISAs of P8 variants combining mutations from different opti-P8 molecules. hGH was fused to wt P8 (wt), an opti-P8 (1a, 2a, or 3a), or P8 variants combining opti-P8 variants (e.g., 1a+2a combines 1a residues 1 through 10 with 2a residues 11 through 20 and wt P8 residues 21 through 50). Sequences of 1a, 2a, and 3a are shown in Figure 1. Assays were performed as described in Figure 2.

After modifying the linker fused to wt P8, we produced a library of P8 proteins fused to the best linker to further explore the relationship between the linker and the phagemid P8 protein. hGH was linked through linker selectant L1 to libraries of P8 variants with mutations in either P8 residues 1 through 10 or P8 residues 11 through 20 (Fig. 4). The libraries were sorted for maximized hGH display, and the resulting P8 variants (Fig. 1) slightly increased hGH display levels (Fig. 5). The less dramatic improvements in protein display levels indicate that we have neared the maximum improvement attainable through modifications to phagemid P8.

To examine the role of linker length on protein display levels, libraries with different linker lengths were constructed, fused to streptavidin (SAV), and selected for optimized SAV display. In this case, SAV was fused to an opti-P8 (2f). The libraries of 5-, 10-, 15-, 20-, and 25-residue linkers employed the restricted diversity strategy described above and were sorted separately (Fig. 4). Only selectants from the library of 5-residue linkers reduced SAV display levels, which demonstrates a minimal size requirement for linkers between SAV and P8. Linkers best able to display SAV on the surface of phage were 15 residues in length, which resulted in a twofold increase in SAV display (data not shown).

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14												
	L1	D	G	S	N	S	T	H	P	H	R	H	N	R	R											
	L2	T	A	R	H	A	N	D	N	D	G	A	H	R	P											
	L3	T	H	P	N	P	R	N	A	A	G	P	A	P	G											
	L4	H	R	N	G	T	D	P	G	G	P	R	A	R	H											
	L5	A	P	R	D	T	T	A	H	R	H	T	H	R	H											
	L6	P	R	S	A	R	S	R	N	T	N	D	R	H	D											
	L7	T	A	P	D	R	S	S	N	D	A	H	A	P	T											
	L8	G	S	P	S	N	P	G	A	R	T	R	A	G	T											
L9	G	H	A	G	H	P	H	R	P	R	H	P	A	R												
B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
	L10	H	A	S	D	D	A	A	R	D	G	R	S	D	N	N	R	G	S	N	G	S	D	S	S	S
	L11	S	H	A	G	N	D	A	G	R	A	R	T	N	H	S	D	G	P	H	G	H	S	S	P	R
	L12	S	A	G	S	D	S	A	R	H	T	A	P	R	S	P	A	S	A	A	N					
	L13	A	P	S	S	A	G	N	D	P	D	R	S	R	S	D	A	R	G	T	G					
	L14	D	G	S	P	N	G	G	R	G	H	N	D	N	P	P	R	G	H	A	P					
	L15	S	A	S	A	D	S	S	R	T	A	A	R	P	P	G	P	G	T	A	S					
	L16	R	S	A	A	G	R	D	A	G	R	D	R	P	A	G	S	S	G	S	H					
	L17	S	G	S	P	A	N	A	P	G	H	H	S	H	H	D	A	R	S	G	P					
	L18	R	A	D	H	G	S	R	A	S	H	D	A	S	R	R										
	L19	H	A	G	A	D	A	D	R	S	S	N	T	D	D	G										
	L20	A	S	R	T	D	A	A	R	D	A	T	A	S	R	P										
	L21	T	G	N	R	T	D	R	A	P	P	A	S	S	P	D										
	L22	P	N	A	R	G	A	N	R	T	A	G	S	T	A	S										
	L23	R	A	S	S	D	A	A	R	P	P	S	S	N	G	A										
	L24	A	R	A	R	T	A	H	H	D	R															
	L25	A	N	S	H	A	A	H	G	T	S															
	L26	T	P	G	H	G	H	P	H	P	D															
	L27	R	G	G	R	A	P	H	S	S	A															
	L28	A	G	R	G	T	S	S	T	R	G															
	L29	P	R	H	D	H	H	P	A	H	D															
	L30	D	R	G	R	T	N	R	T	D	T															
	L31	A	R	A	N	R																				
	L32	R	H	N	R	R																				
	L33	D	H	S	S	A																				
	L34	A	R	G	P	T																				
	L35	H	T	P	G	A																				
	L36	N	S	G	G	D																				
L37	R	T	T	S	N																					

Fig. 4. Amino acid sequences of linkers optimized for the display of proteins fused to P8. Linkers were selected for increased display of (A) hGH or (B) SAV.

Discussion

Wild-type P8 inefficiently displays protein fusions on the surface of phage. This was apparent from early examples of protein display on P8 (reviewed by Wilson & Finlay, 1998), from direct comparisons of protein display on both P8 and P3 (Kretzschmar & Geiser, 1995) and from our studies (Sidhu et al., 2000b). The large size of the protein fusion may appear to be an obvious cause of low protein display levels. However, steric bulk alone fails to account for low level display by P8-fusion proteins, as X-ray diffraction and modeling studies suggest that P8 can accommodate large polypeptide fusions without steric hindrance (Malik et al., 1996). Here, we provide empirical evidence that the steric bulk of the displayed protein does not limit fusion protein display by P8. Without changing the size or sequence of the fusion protein, protein display levels were increased dramatically, through mutations in the first 30 residues of the fusion P8.

The linker between fusion protein and phagemid P8 provides a spacer to mitigate disruption of phage assembly by bulky fusion proteins (Nakayama et al., 1996). Considerations for ideal linkers include flexibility, resistance to proteolysis, and length between the phage particle and the fusion protein. Mutagenesis and selection of linkers with enhanced display of fusion proteins

simultaneously optimized these variables. Selected linkers, unlike selected opti-P8s, feature a nearly even distribution of amino acids, with no preferred amino acids in any position. Lack of preferred amino acids in specific positions discounts the possibility of enhanced display through specific binding interactions between the phage coat and the linker. Many different possible interactions, in the form of diverse amino acid substitutions, were observed in each position with roughly equivalent effects on protein display levels.

P8 transitions through at least three different binding configurations. After synthesis, P8 forms a homodimer (Haigh & Webster, 1998), interacts with the phage-extruding pore (Russel, 1993), and packages viral single stranded DNA, while forming a right-handed coiled coil phage coat (Marvin et al., 1994). The opti-P8s presumably transition through these interactions too; however, as protein fusions these incorporate into the phage coat more effectively than wt P8, and thus enhance fusion protein display levels. Protein display by opti-P8s could result from improved contacts with many binding partners. Although some effect on P8 homodimerization or P8 interactions with the pore cannot be ruled out, homodimer formation (Haigh & Webster, 1998) and pore interactions (Russel, 1993) are expected to involve the C-terminus of the P8 molecule, a region distant from our mutations to residues 1 through 30.

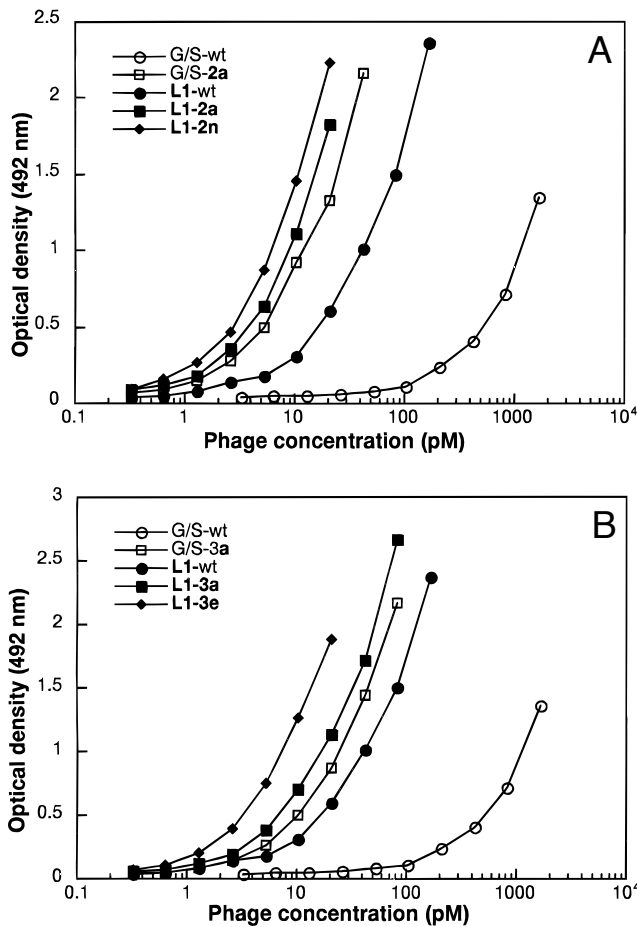


Fig. 5. Phage ELISA of hGH display with optimized linkers. hGH was fused to wt P8 (wt) or an opti-P8 with mutations in (A) residues 11 through 20 (either 2a or 2n) or (B) residues 21 through 30 (either 3a or 3e). hGH was fused to the phagemid P8 by either a Gly/Ser-rich linker sequence (G/S) or the hGH display-optimized linker sequence L1. Assays were performed as described in Figure 2.

Since the opti-P8s are stable scaffolds for protein display and result in normal phage propagation and infectivity (Sidhu et al., 2000b), it is reasonable to expect the opti-P8s to integrate normally into the mature phage coat. Within the mature phage coat, the first five residues of each P8 molecule are solvent exposed (Marvin et al., 1994), while residues beyond approximately residue 30 are buried within the phage coat. Residues 6 through 29 form an amphipathic helix with a hydrophilic, solvent exposed face and a hydrophobic face buried through contacts with other P8 molecules in the phage coat.

Despite sequence variation among opti-P8s, three groups of residues emerge. The first group of residues (Ala7, Ala10, Leu14, and Ala18) generally preferred wild-type sequence during selection for maximized protein display (Fig. 1). Interestingly, these residues cluster along one hydrophobic face of the P8 alpha helix (Fig. 6), a face thought to interact with adjacent, wt P8 molecules of the phage coat (Marvin et al., 1994). The facial alignment of these small, hydrophobic residues and their resistance to mutation within the context of the phage genome (mutagenesis of all 2,700 copies of P8) has been described previously. The small side chains at these positions likely enable close packing of P8 molecules during

oligomerizing phage coat assembly (Williams et al., 1995; Papavoine et al., 1998).

The hydrophobic face defined by residues Ala7, Ala10, Leu14, and Ala18 remains important for incorporation of the phagemid P8 fusion into the phage coat. During selection for maximized protein display, these positions either remained wild-type or accepted only conservative substitutions with very similar side chains (Fig. 1). This suggests that incorporation into the phage coat is the primary selection factor for high level protein display. Conservation of these residues was sufficient to allow high copy display, even with 25 out of 30 N-terminal P8 residues mutated from wild-type. Residues proximal to these small hydrophobic residues generally showed consensus for amino acids with hydrophobic side chains.

The second group of residues contains mutations that expand the conserved hydrophobic face of the P8 alpha helix (P6F, S17I, and A25L) (Fig. 6B). Residues in this group generally converge to amino acids with hydrophobic side chains. Expansion of the conserved hydrophobic face may enhance incorporation of the phagemid P8 and fusion protein into the phage coat, through favorable packing interactions with other P8 molecules. Point mutations of these residues significantly impaired protein display levels and were the most critical single residue substitutions in Figure 2. A few selectants defied the general preference for hydrophobic residues in these positions. However, we have demonstrated how multiple mutations can additively accumulate protein-display enhancing effects (Fig. 2D); such additive effects could compensate for the loss of a key hydrophobic residue in a few selectants.

Residues distal from the conserved hydrophobic face of the P8 alpha helix compose the final category of P8 mutations. This distal face (Fig. 6C) forms the outer surface of the virion, and in wt P8, consists of mainly hydrophilic side chains. As might be expected for mutated surface residues, we observed little consensus for amino acid substitutions and found abundant diversity in amino acid selection. One observed trend was selection for positively charged residues along this face in the case of SAV and negatively charged side chains for hGH-fused P8 variants. This protein specific charge preference might reflect selection for charge-charge interactions or repulsions between the phagemid P8 and the fusion protein.

Combination of back mutations additively decreased hGH display levels (Fig. 2D). For example, combination of I17S with other mutations decreased hGH display to levels that are approximately the sum of each individual mutation. Thus, protein display-enhancing mutations may act independently, with only a few, key mutations making significant contributions. Our work identifies key determinants for enhanced protein display and future work focusing on these residues may yield further improvements in protein display levels.

In summary, we show that the requirements for P8 incorporation into the phage are remarkably lax in the phagemid system. Perhaps because each phage particle contains several thousand wt P8 molecules, heavily mutated variants can be readily incorporated as minor coat components. Within the first 30 residues of P8, only four wt residues remained conserved during selection for fusion protein incorporation. The small, aliphatic side chains at these positions form a hydrophobic core that could pack against adjacent P8 molecules in the phage coat. Increased incorporation of coat protein fusions was achieved through mutations that increased the hydrophobicity surrounding the conserved core. Promiscuous incorporation of proteins into the filamentous bacteriophage has two important implications. For practical applications, it enables the

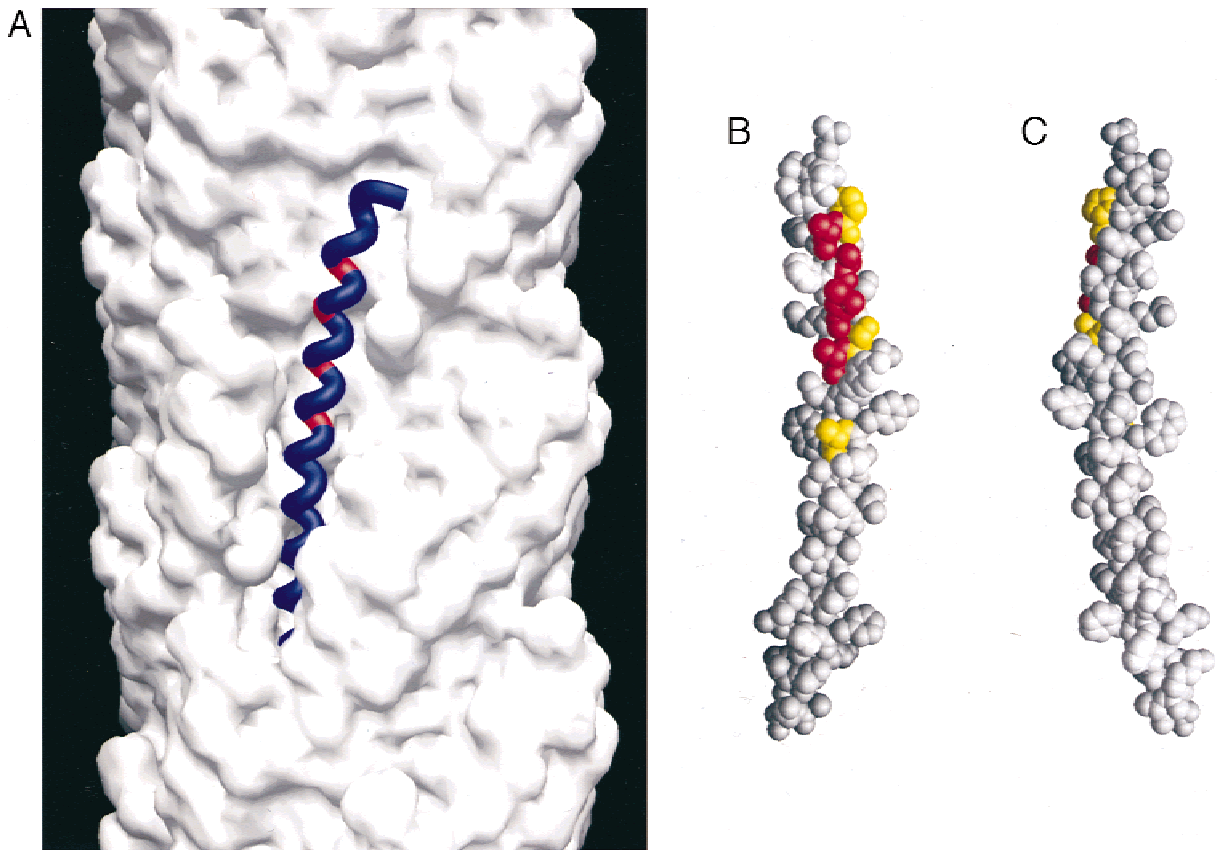


Fig. 6. Structural models of opti-P8 molecules within the phage coat. **A:** The P8 alpha helix (blue) forms key interactions with other P8 molecules of the mature phage coat (white). The opti-P8 variants conserve residues of the coat-buried, hydrophobic core (Ala7, Ala10, Leu14, and Ala18; shown in red). **B:** Hydrophobic substitutions for the wt P8 residues shown in yellow (Pro6, Ser17, and Ala25) enhance protein display levels, as demonstrated by large decreases in hGH display following back mutation (Fig. 2). Display enhancing mutations at these sites expand the hydrophobic patch formed by Ala7, Ala10, Leu14, and Ala18 (shown in red). **C:** Rotation of the P8 alpha helix by 180° reveals the unconserved hydrophilic face. Mutations back to wt P8 along this face did not significantly influence protein display levels. Figures were produced using the Grasp software (Nicholls et al., 1991), and depict wt P8 of the Ff phage coat (PDB entry 1IFJ). M13 P8 differs from Ff P8 in only one position (Asn12 in M13 P8 is an Asp in Ff P8).

engineering of coat protein variants better suited for phage display, while in natural evolution, it could enable phage to readily evolve new coat proteins and thus new functions.

Materials and methods

Materials

Reagents for DNA sequencing were from United States Biochemicals (Cleveland, Ohio). Enzymes were from New England Biolabs (Beverly, Massachusetts). Maxisorp immunoplates were from NUNC (Roskilde, Denmark). *E. coli* XL1-Blue and M13-VCS were from Stratagene (La Jolla, California). Bovine serum albumin (BSA), Tween-20, and *o*-phenylenediamine dihydrochloride were from Sigma (St. Louis, Missouri). Horse radish peroxidase/anti-M13 antibody conjugate was from Pharmacia Biotech (Piscataway, New Jersey).

Oligonucleotides

DNA degeneracies are represented in the IUB code (K = G/T, N = A/C/G/T, R = A/G, S = G/C, V = A/C/G, W = A/T, Y = C/T). Oligonucleotide sequences were as follows:

g8stop2: GATCCCGCAAAGCGGCCTGATGATCCCTGCAAGCCT
CAGCG

g8stop3: CAAGCCTCAGCGACCGAATGATGAGGTTATGCGTGGG
CGATG

g8V2: GATCCCGCAAAGCGGCCNWTNASRNTNYTNASRNRNT
RNRNTNASTATATCGGTTATGCGTGG

g8V3: CAAGCCTCAGCGACCGAANWCNKNWNCNYTNGNY
TNKGNWTNWTGTCATTGTGCGGCAACTATC

hGH-Lstop: GGTGGAGGATCCGGGAGCTGATGAGCCGAGGGTGAC
GATCCC

hGH-L14: GAGGGCAGCTGTGGCTTCGGTGGCGGTVVCVVCVVC
VVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVC
GAGGGTGACGATCCC

SAV-Lstop: CACCAAGGTGGTCTAGAGCTAATAATAAGCCGAGGG
TGACGATCCC

SAV-L5: CACCAAGGTGGTCTAGAGCVVCVVCVVCVVCVVCVVC
GAGGGTGACGATCCC

SAV-L10: CACCAAGGTGGTCTAGAGCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVCGCCGAGGGTGACGATCCC

SAV-L15: CACCAAGGTGGTCTAGAGCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVCGCCGAGGGTGACGATCCC

SAV-L20: CACCAAGGTGGTCTAGAGCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVCGCCGAGGGTGACGATCCC

SAV-L25: CACCAAGGTGGTCTAGAGCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVVCVCGCCGAGGGTGACGATCCC

Construction of linker libraries

Phagemids designed for the display of hGH (phagemid pS1607) or SAV (phagemid pW277e) fused to the N-terminus of P8 have been described previously (Sidhu et al., 2000b). In both cases, the heterologous protein was linked to P8 by a Gly/Ser-rich linker sequence (QSGGSGSSS). Libraries were designed to replace the codons encoding the Gly/Ser-rich sequence with varying numbers of degenerate VVC codons (VVC encodes Ala, Arg, Asn, Asp, Gly, His, Pro, Ser, or Thr).

Libraries were constructed using a modified version (Sidhu et al., 2000a) of a previously described method (Lowman, 1998). For hGH display, oligonucleotide *hGH-Lstop* was used with pS1607 as a template to introduce stop codons within the linker sequence between hGH and P8, using the method of Kunkel et al. (1987). The resulting phagemid was used as a template for a second round of Kunkel mutagenesis with oligonucleotide *hGH-L14*. This resulted in a library of phagemids encoding hGH linked to P8 by random 14-residue polypeptides. The diversity of this library was 1.8×10^{10} . For SAV display, a derivative of PW277e, which encoded SAV fused to a SAV display optimized P8 variant 2f, was used as the parent template for library constructions. Oligonucleotide *SAV-Lstop* was used to introduce stop codons within the linker sequence between SAV and 2f. The resulting phagemid was used as a template in a second round of Kunkel mutagenesis with oligonucleotide *SAV-L5*, *SAV-L10*, *SAV-L15*, *SAV-L20*, or *SAV-L25*. The resulting libraries of phagemids encoded SAV linked to P8 (2f) by random polypeptides containing 5, 10, 15, 20, or 25 residues, respectively. The diversities of these libraries were as follows: *SAV-L5*, 1.5×10^{10} ; *SAV-L10*, 9.8×10^9 ; *SAV-L15*, 1.3×10^{10} ; *SAV-L20*, 1.1×10^{10} ; *SAV-L25*, 6×10^9 .

Construction of mutant P8 libraries

Libraries of P8 mutants fused to hGH were constructed as described previously (Sidhu et al., 2000b). hGH was linked to P8 by an optimized linker sequence (L1; Fig. 4). Oligonucleotide *g8stop2* or *g8stop3* was used to introduce stop codons into zone 2 (P8 residue 11–20) or zone 3 (P8 residues 21–30), respectively. Oligonucleotide *g8V2* or *g8V3* was used to construct a library of P8 variants with mutations in zone 2 or zone 3, respectively. The diversity of each library was 1.7×10^{10} .

Selection for fusion protein display

Phage from the hGH-P8 libraries described above were cycled through rounds of binding selection with the human growth hor-

mone binding protein (hGHbp) (Fuh et al., 1990) coated on 96-well Maxisorp immunoplates as the capture target. All libraries were sorted separately. Phage were propagated in *E. coli* SS-320 cells (Sidhu et al., 2000a) with M13-VCS helper phage. After five rounds of binding selection, individual phage were isolated and analyzed in a phage ELISA by capturing the hGH-phage with hGHbp immobilized on a plate, and detecting bound phage (see below). Phage exhibiting strong signals in the phage ELISA were subjected to sequence analysis.

In a similar fashion, the SAV-P8 libraries were cycled through rounds of binding selection with anti-SAV polyclonal antibody as the capture target. Phage were propagated in the *SupE E. coli* strain XL1-Blue in which the amber stop codon is suppressed as glutamine (Bullock et al., 1987).

Site-directed mutagenesis

Mutagenesis was performed using the method of Kunkel et al. (1987). Mutations were confirmed by DNA sequencing.

Phage ELISAs for determining relative levels of fusion protein display

Phage ELISA protocols were adapted from a previous work (Pearce et al., 1997). Cultures of *E. coli* XL1-Blue harboring phagemids were grown for 8 h at 37 °C in 1 mL of 2YT, 50 μg/mL carbenicillin, 10 μg/mL tetracycline. The cultures were transferred to 30 mL of the same media (supplemented with M13-VCS helper phage (10¹⁰ phage/mL) and IPTG at the appropriate concentration) and grown overnight at 37 °C. Phage were harvested from the culture supernatant by precipitation twice with PEG/NaCl (Lowman, 1998) and resuspended in 1 mL of phosphate-buffered saline, 0.2% BSA, 0.1% Tween 20 (BSA blocking buffer). Phage concentrations were determined spectrophotometrically ($\epsilon_{268} = 1.2 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$).

Maxisorp immunoplates (96-well) were coated with an anti-hGH monoclonal antibody (MCD; Jin et al., 1992) for 2 h at room temperature (100 μL at 5 μg/mL in 50 mM carbonate buffer, pH 9.6). The plates were then blocked for 1 h with 0.2% BSA in phosphate-buffered saline (PBS) and washed eight times with PBS, 0.05% Tween 20. Phage particles were serially diluted into BSA blocking buffer and 100 μL were transferred to coated wells. After 1 h, plates were washed eight times with PBS, 0.05% Tween 20, incubated with 100 μL of 1:3000 HRP/anti-M13 conjugate in BSA blocking buffer for 30 min, and then washed eight times with PBS, 0.05% Tween 20 and two times with PBS. Plates were developed using an *o*-phenylenediamine dihydrochloride/H₂O₂ solution (100 μL), quenched with 2.5 M H₂SO₄ (50 μL), and read spectrophotometrically at 492 nm.

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