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PhiXing-it, displaying foreign peptides on bacteriophage ΦX174

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ABSTRACT

Although bacteriophage φX174 is easy to propagate and genetically tractable, it is use as a peptide display platform has not been explored. One region within the φX174 major spike protein G tolerated 13 of 16 assayed insertions, ranging from 10 to 75 amino acids. The recombinant proteins were functional and incorporated into infectious virions. In the folded protein, the peptides would be icosahedrally displayed within loops that extend from the protein's β-barrel core. The well-honed genetics of φX174 allowed permissive insertions to be quickly identified by the cellular phenotypes associated with cloned gene expression. The cloned genes were easily transferred from plasmids to phage genomes via recombination rescue. Direct ELISA validated several recombinant virions for epitope display. Some insertions conferred a temperature-sensitive (*ts*) protein folding defect, which was suppressed by global suppressors in protein G, located too far away from the insertion to directly alter peptide display.

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Introduction

Inexpensive and quick to propagate, bacteriophages are attractive platforms for multivalent peptide display (Brown et al., 2002; Clark and March, 2006; Danner and Belasco, 2001; Ren and Black, 1998; Shivachandra et al., 2007; Smith, 1985; Sternberg and Hoess, 1995; Tumban et al., 2011). Numerous systems based on bacteriophages T4, T7, λ, MS2, PP7, Qβ, and filamentous phage have been developed for diverse applications, which include the selection of peptide or protein binders through phage display, the development of novel imaging reagents, biosensors, bio-nanoconjugate applications, and for antigen display platforms for serological assays, epitope mapping, and vaccine development (Henry et al., 2015; Lee et al., 2013; Levy et al., 2007; O'Rourke et al., 2015; Seker and Demir, 2011). Peptide display from viral capsid or virus-like particle (VLP) scaffolds is empirical by nature; it is impossible to predict whether a given peptide will be tolerated or will perform successfully in any given application. Although many systems have been described, no one platform is perfect for the display of every peptide and every technology. Differences in peptide valency, arrangement, geometry, and conformation of display may have profound and unanticipated differences in performance (Jagu et al., 2015; Krause et al., 2006; Wu et al., 2015). Finally, for vaccine applications, display of a particular peptide antigen on multiple VLP display platforms may be necessary to avoid unwanted immunity towards the carrier after multiple immunizations (Jegerlehner et al., 2010). Further exploration of novel peptide display platforms is therefore warranted.

Here, we sought to expand the phage-based peptide display options by investigating the utility of bacteriophage φX174, a very genetically tractable system (Chen et al., 2007; Cherwa et al., 2009). Although φX174 structure, assembly, genetics, and replication have been extensively studied, this well-characterized system has never been explored as a peptide-display platform. φX174 is the canonical microvirus species within the *Microviridae*. Although the (+) single-stranded DNA genomes are small (~5.4–6.1 kilobases), the use of overlapping reading frames allow them to encode as many as 12 genes (Doore et al., 2014). The genes encoding the scaffolding, DNA replication, and lysis functions can

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overlap extensively, whereas the structural proteins are encoded in regions dedicated to four single genes.

Virions display $T=1$ icosahedral symmetry. Along with the circular genome, the capsid's interior contains 60 copies of the DNA binding protein J and 10–12 copies of the DNA pilot protein H, which emerges from the capsid as a tube on the cell surface (Sun et al., 2014). The exterior is composed of 60 copies each of the viral coat F and major spike G proteins. Both proteins consist of eight anti-parallel β-strands arranged into a classical β-barrel motif (McKenna et al., 1994). Nascent spike proteins quickly fold and accumulate as soluble pentamers inside infected cells before being assembled into soluble sub-virion capsomeres (Cherwa et al., 2008). Unlike the viral coat protein, which forms extensive protein–protein interactions with both the external and internal scaffolding proteins throughout morphogenesis, the upper and side surfaces of G protein pentamers are solvent exposed, forming the 12 prominent spikes that decorate the icosahedral 5-fold axes of symmetry in both virion and procapsid X-ray structures (Dokland et al., 1999, 1997; McKenna et al., 1994). Thus, protein G appeared to be the most suitable for peptide display. In this study, we identified an exposed surface loop in the major spike protein that could tolerate a wide variety of amino acid insertions ranging from 10-75 amino acids.

Results and discussion

Identification of a φX174G protein insertion loop suitable for peptide display

Due to the protein's prominent, icosahedrally-ordered surface display and the lack of scaffolding protein interactions during assembly (Dokland et al., 1999; McKenna et al., 1994), the major spike protein was identified as the optimal protein for peptide display (Fig. 1A). Codons 20–29 of the HPV16 L2 gene were used as the model peptide for these initial pilot experiments. This region encodes a conserved neutralizing epitope: $_{20}$KTCKQAGTCP$_{29}$ (Gambhira et al., 2007; Rubio et al., 2011) that forms a disulfide-bonded loop in the L2 minor capsid protein of mature Human Papillomavirus Type 16 virions (Campos and Ozbun, 2009). L2$_{(20\text{-}29)}$ encoding DNAs were flanked by *Bam*HI and *Bsp*EI linkers to facilitate cloning. The linker-encoded Gly-Ser and Ser-Gly

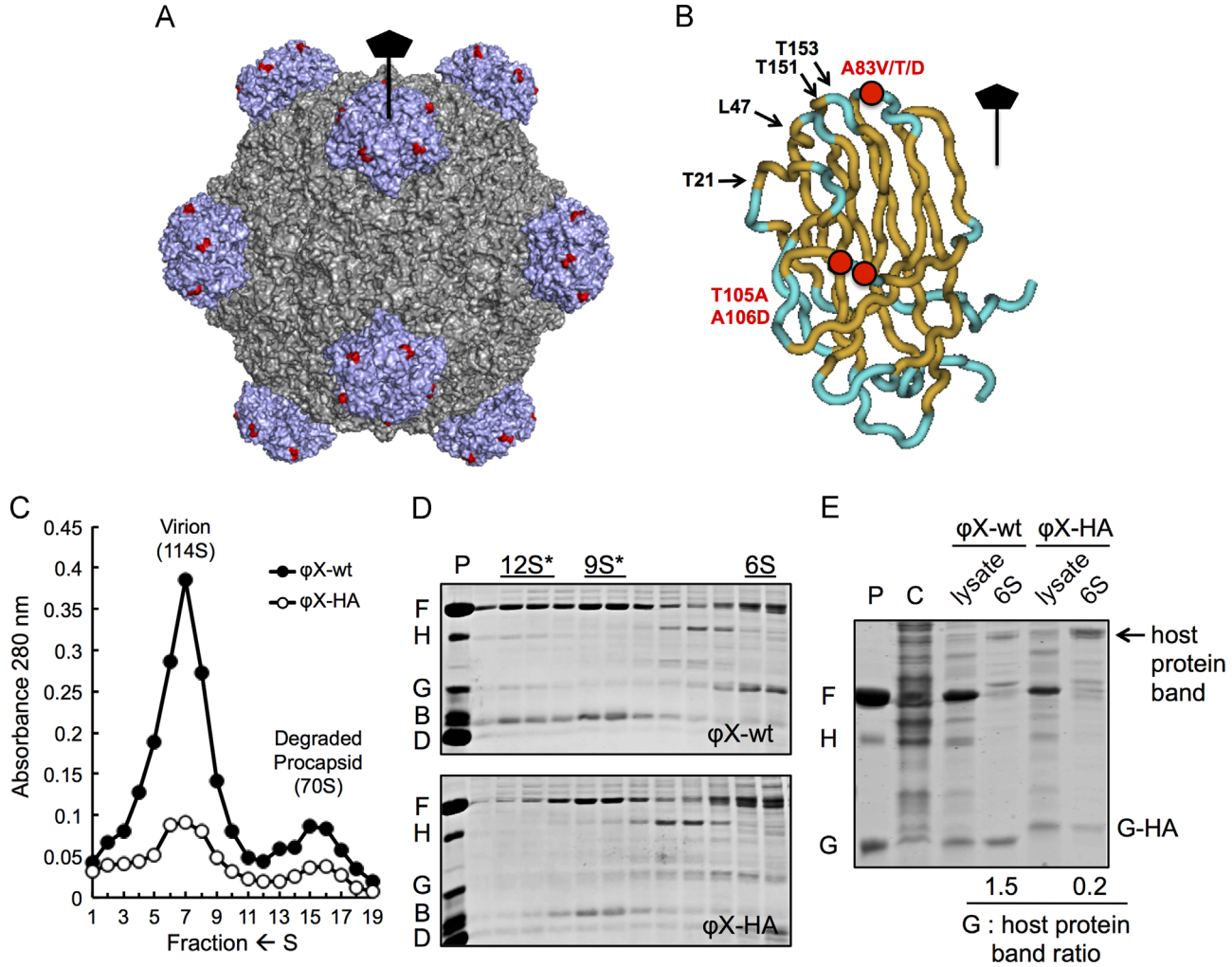

Fig. 1. Structure of bacteriophage φX174. (A) Isosurface tracing of the φX174 atomic structure. Spike protein G and coat protein F are, respectively, depicted in blue and grey. The location of foreign antigen display at Thr21 is highlighted in red. The 5-fold axis of symmetry is marked with a pentagon. (B) The atomic structure of the spike protein G. β-strands are depicted in yellow and loops are depicted in blue. The location of the insertion sites after amino acids 21, 47, 151, and 153 are indicated with black arrows. The location of the second site suppressors at amino acids Ala83, Thr105, and Ala106 are depicted with red dots. (C) Sedimentation profiles of assembled φX174 wild type and φX-HA particles: degraded procapsids (70S) and virions (114S) from extracts of infected cells at 42 °C. (D) SDS-PAGE of small particle (6S-12S) gradient fractions of a wild-type φX174 infection (upper blot) and a φX-HA infection (lower blot). (E) Side by side comparison of SDS-PAGE of infected cell lysates and 6S fractions. Procapsid marker (P), depicting proteins F, H, and G, and uninfected cell lysate (C) are shown as well. G spike protein bands and the host cell normalization protein band are indicated with arrows (see text for details).

Table 1
Complementation and recombination viability of *am(G)Q33* with amino acid insertion clones.

Plasmid characteristics				Recombinant phage characteristics	
Cell line	Cell viability[a]	Complementation of *am(G)Q33* [b]	Recombination rescue	Name and phenotype	Suppressors
BAF5(*supE*)[b]	NA[c]	1.0	NA	NA	NA
C122 (*sup*$^-$)[b] BAF30 with	NA	$<5\times10^{-6}$	NA	NA	NA
pφXG	±	0.8	NA	NA	NA
pφXG21-L2$_{(20\text{-}29)}$	−	NA	5×10^{-5}	φX-L2, weak *ts*[d]	A83D[e],V and T T105A, A106D
pφXG47-L2$_{(20\text{-}29)}$	+	$<5.0\times10^{-6}$	$<2.0\times10^{-7}$	NA	NA
pφXG151-L2$_{(20\text{-}29)}$	+	$<5.0\times10^{-6}$	$<2.0\times10^{-7}$	NA	NA
pφXG153-L2$_{(20\text{-}29)}$	+	$<1\times10^{-3}$	$<2.0\times10^{-7}$	NA	NA
pφXG21-HA	±	1.0	5.0×10^{-5}	φX-HA, *ts* insert changes[f] with A83V or A106D	
pφXG21-myc	−	NA	8.6×10^{-5}	φX-myc	NA
pφXG21-BTAG	−	NA	6.5×10^{-5}	φX-BTAG	NA
pφXG21-L2$_{(16\text{-}36)}$	+	$<8\times10^{-4}$	$<7.8\times10^{-7}$	NA	NA
pφXG21-PSTCD	±	0.3	See text	See text	NA
pφXG21-M2a	−	NA	2.1×10^{-4}	φX-M2a	NA
pφXG21-M2b	−	NA	2.0×10^{-4}	φX-M2b, *ts*	A83V
pφXG21-M2c	−	NA	3.0×10^{-4}	φX-M2c, weak *ts*	A83V
pφXG21-CSa	−	NA	6.0×10^{-5}	φX-CSa, weak *ts*	A83D
pφXG21-CSc	+	NA	$<5.1\times10^{-6}$	NA	NA
pφXG21-CSd	+	NA	$<3.3\times10^{-6}$	NA	NA
pφXG21-CSe	−	NA	3.0×10^{-5}	φX-CSe	NA

Not applicable.

[a] Indicates whether induction of the clone gene prevented lawn formation. Symbols: +, no effect on lawn formation, ±, a moderate effect, – complete inhibition of cell growth.

[b] Assay titer/most permissive titer.

[c] BAF5 cell line contains a tRNA informational suppressor, inserting glutamine during protein synthesis. C122 does not contain an informational suppressor.

[d] A weak *ts* phenotype was defined as a plating efficiency between 0.1–1.0 at 42 °C compared to 33 °C and plaques displayed a pin-prick morphology. Phage with other ts phenotypes yielded 42 °C plating efficiencies of 10^{-4} or lower. When no phenotype is given the phenotype closely resembled wild-type.

[e] All suppressors were intragenic. Nomenclature: amino acid substitutions in the protein G: wild-type amino acid, position, substituted amino acid. Thus, A83D, an alanine to a aspartic acid at residue 83.

[f] These phages contained mutations within the HA insert as well as in G protein. The changes in the insert were Y5F, Y10H, D9G. Numbering of the insert is the same as given in Table 2.

Table 2
Amino acid insertions placed at position Thr21 of G protein.

Insert and description		Length	Charge	Sequence	Tolerated[a]
L2$_{(20\text{–}29)}$	HPV16 L2	14	+2	GSKTCKQAGTCPSG	Yes
HA	epitope tag	13	−2	GSYPYDVPDYASG	Yes
	Selected variant	13	−2	GSYP**F**DVPDYASG	Yes
	Selected variant	13	−1	GSYPYDVP**G**YASG	Yes
	Selected variant	13	−2	GSYPYDVPD**H**ASG	Yes
myc	Epitope tag	14	−3	GSEQKLISEEDLSG	Yes
BTAG	Epitope tag	10	0	GSQYPALTSG	Yes
L2$_{(16\text{–}36)}$	HPV16 L2	25	+2	GSTQLYKTCKQAGTCPPDIIPKVSG	No
PSTCD	Biotin ligase substrate	75	−2	GSGEGEIPAPLAGTVSKILVKEGDTP KVSGTVLVLEAMKMETEINAPTDGK VEKVLVKERDAVQGGQGLIKGGSG	Yes
M2a	Influenza A M2 protein	13	−2	GSSLLTEVETPSG	Yes
M2b	Influenza A M2 protein	15	−2	GSHYSLLTEVETPSG	Yes
M2c	Influenza A M2 protein	15	−1	GSSLLTEVETPTRSG	Yes
CSa	*P. falciparum* CS protein	16	0	GSNANPNANPNANPSG	Yes
CSc	*P. falciparum* CS protein	24	0	GSNANPNANPNANPNANPNANPSG	No
CSd	*P. falciparum* CS protein	28	0	GSNANPNANPNANPNANPNANPNANPSG	No
CSe	*P. falciparum* CS protein	28	−1	GSNANPNVDPNANPNANPNANPNANPSG	Yes

[a] Indicates whether the insertion was tolerated within the phage genome or yielded a complementing clone. The PTSCD insertion was too large to be moved into the genome, see text for details.

sequences may also provide protein flexibility. The L2$_{(20\text{–}29)}$ epitope was inserted into four different locations within a complementing, plasmid-based, φX174 major spike gene (Table 1). This positively charged 14-mer epitope would theoretically be displayed after positions Thr21, Leu47, Thr151, or Thr153, in one of three loops that extend off of the G protein's Swiss jellyroll core (Fig. 1B, Table 2).

Although it is possible to conduct complementation assays with expression plasmids containing the cloned wild-type φX174G gene, induction is somewhat toxic to host cells. Modified gene G induction conferred two distinct phenotypes, neither of which resembled the wild-type control (Table 1). The induction of the cloned φXG21-L2 gene killed host cells, whereas φXG47-L2, φXG151-L2 and φXG153-L2 gene induction resulted in no observable toxicity. Unlike the wild-type

G gene, these recombinant genes failed to complement the φX174 *am(G)Q*33 mutant. The elevated or decreased toxicity levels proved a very reliable readout for G protein functionality (see below).

To determine if altered genes could be placed directly into viable phage genomes, recombination rescue experiments were performed. φX174 *am(G)Q*33 was plated on plasmid containing cells without IPTG induction. Two genetic mechanisms will allow plaque formation; 1) direct reversion of the *am* phenotype, yielding wild-type φX174 or 2) recombination with the cloned gene, yielding a genomic gene with an in-frame insertion. To distinguish between these alternatives, plaques were screened for altered phenotypes by stabbing them into host cell lawns incubated overnight at 33 °C and 42 °C. In all instances, it was possible to distinguish between wild-type and insertion-containing φX174 strains. Wild-type φX174 produced a large clearing surrounded by a thick halo, whereas the recombinant strains produced small clearings with sharp edges. This phenotypic correlation was verified by DNA sequencing, and was observed with other recombinant strains (see below). At position Thr21, viable recombinants were recovered at a frequency of 5.0×10^{-5}. Recombinants were not recovered at Leu47, Thr151, or Thr153 (frequency $< 10^{-7}$). These data suggest that only the loop at Thr21 could tolerate the insertion of the neutralizing $L2_{(20\text{-}29)}$ epitope (Table 1).

The identified φX174G protein insertion loop can tolerate many inserted peptides

To determine if other insertions were tolerated at Thr21, a variety of peptide encoding DNAs were cloned as described above (Tables 1 and 2). We tested a diverse set of model epitope tags and fusion domains: hemagglutinin (HA) epitope, myc epitope, the Bluetongue virus VP7 epitope tag (BTAG), and the *P. Shermanii* transcarboxylase domain (PSTCD); as well as *bona fide* neutralizing epitopes from HPV16 L2 (RG-1), Influenza A virus M2 protein (M2), and the circumsporozoite protein (CS) from *Plasmodium falciparum* (Gambhira et al., 2007; Murtif et al., 1985; Reddy et al., 2000; Wang et al., 1996; Young et al., 2012; Zavala et al., 1985; Zebedee and Lamb, 1988). These inserts encompass a wide variety of sizes, charges, and side chain chemistries (Table 2).

Cloned genes were assayed for elevated host cell toxicity upon induction and the ability to complement φX174 *am(G)Q*33. As can be seen in Table 1, three phenotypes were associated with the induced clones: complementing (plating efficiencies > 0.1), non-complementing (plating efficiencies $< 10^{-2}$) with no associated host-cell toxicity, or elevated host cell toxicity. Recombination rescue experiments were performed as described above. In all cases, recombinant phages were recovered using the complementing and/or toxic genes with small inserts. The modified gene with the 75 residue PSTCD domain complemented the φX174 mutant. The insertion would add 228 nucleotides to the viral genome, which exceeds the stringent 102% limit for genome encapsidation (Aoyama and Hayashi, 1985). Thus, recombination rescue experiments were not performed. In total, 13/16 inserts were tolerated, implying high permissibility for foreign peptide insertion at Thr21.

Correlation between the toxicity of induced cloned genes and modified G protein function

Cloned gene induction resulted in three phenotypes vis-à-vis host cell viability. At 33 °C, cells harboring the wild-type φX174G gene can tolerate IPTG concentrations of ~1.0 mM, which leads to the efficient complementation of *am(G)* mutants. 5.0 mM IPTG concentrations kills cells. When modified cloned gene induction resulted in a similar phenotype, complementation and recombination rescue were also observed. Thus, these modified G proteins assume a near wild-type fold. Induced genes that had no effect on host cell viability neither complemented nor yielded recombinant phages. If host cell toxicity is a function of the folded protein, these recombinant proteins are likely unable to fold. Lastly, the induction of some clones was too toxic for complementation assays. In all instances, these clones yielded recombinant phages. Although the exact mechanism conferring elevated toxicity is not known, it appears to serve as a reliable and convenient measure of whether an insert will ultimately result in a viable recombinant phage.

Recovered recombinant φX174 particles exhibit varying phenotypes

Some recombinant viruses, φX-HA and φX-L2, exhibited temperature-sensitive (*ts*) phenotypes, growing poorly above 37 °C (Table 1). To investigate the nature of this defect, φX-HA phage generated at 33 °C were assayed for particle stability, host cell attachment kinetics, and eclipse kinetics at 42 °C. At the restrictive temperature, φX-HA did not differ significantly from wild-type (data not shown). To investigate possible protein folding and/or particle assembly defects, lysis resistant cells were infected with either wild-type φX174 or φX-HA at 42 °C. After a 4-hour incubation, extracts were prepared and analyzed by rate zonal sedimentation. Particles, regardless of infectivity, are detected by UV spectroscopy. The sedimentation profiles for complexes with 70-140S values are depicted in Fig. 1C. Mutant virion yields were dramatically lower than wild-type. However, the specific infectivity of the φX-HA 114S peak fraction was not reduced: 5×10^{11} pfu/A_{280} vs. 6.0×10^{11} pfu/A_{280}, respectively. Moreover, significant amounts of off-pathway, uninfectious particles (70S) were not detected (Fig. 1C). These data suggest that the *ts* defect affects protein folding and/or is manifested during early assembly.

The presence of smaller assembly intermediates was examined by SDS-PAGE of gradient fractions within the 6S-12S range, which contain the early assembly intermediates (Fig. 1D). Unlike the wild-type control, 12S* particles were not detected in φX-HA infected cell extracts (Fig. 1C). This particle contains the F, G, B and H proteins in a respective 5:5:5:1 ratio. It is formed by the addition of one G protein pentamer (6S particle) to the 9S* intermediate. 6S gradient fractions, containing soluble G protein pentamers were compared side by side on an SDS-PAGE gel (Fig. 1E). The intensity of the G protein band was normalized to a host protein band, which is present in both cell lysates and 6S fractions and indicated in Fig. 1E. In whole cell lysates, this protein G:host protein ratio measures total cellular G protein, regardless of solubility. Protein G was abundant in both the wild-type and φX-HA whole cell lysates with ratios of 2.7 and 1.6, respectively. The G protein levels in the 6S fraction roughly estimates the amount of folded soluble protein. If mutant 6S particles form but are not incorporated into 12S* particles, they would accumulate in this fraction. Within the 6S fraction, the wild-type protein G: host protein ration is 1.5. By contrast, the φX-HA protein G:host protein ratio is only 0.2. Thus, φX-HA 6S particles appear to be vastly underrepresented when compared to the wild-type control. These data indicate that the HA recombinant G protein folds poorly at 42 °C and is unable to assemble into 6S* pentamers.

Second-site suppressors within G correct for high-temperature growth defects

Suppressors of the *ts* phenotype were selected by plating φX-HA, φX-M2b, φX-M2c, and φX-CSa and φX-L2 at 42 °C, as described in *Materials and Methods*. Seven independent phage preparations were used to isolate suppressors of the φX-L2 *ts* growth defect. The A83V mutation was isolated thrice independently, whereas the A83D mutation was isolated twice independently. Thus, the selection likely approached saturation (Luria and

Delbruck, 1943). Other suppressors were located at Thr105 and Ala106 (Table 1). These substitutions affect two different insertion loops within the G protein (Fig. 1B). In less exhaustive selections, substitutions for A83 were isolated in the φX-M2b, φX-M2c, and φX-CSa backgrounds. The suppressor of the φX-HA *ts* phenotype were located directly within the HA insertion (Tables 1 and 2). However, one of these substitutions HA-D9G was always isolated in conjunction with either the A83V or A106D mutations. These results indicate that most folding defects can be corrected by global suppressors located too far away from the insertion to directly alter antigen display.

Recombinant φX174 virions display foreign antigens

Antibodies against the HPV16-L2 epitope used in these studies can neutralize HPV infections *in vitro* (Gambhira et al., 2007; Rubio et al., 2011), and elicit protective humoral responses in animal studies (Alphs et al., 2008; Jagu et al., 2009). To determine if these previously characterized anti-L2 monoclonal antibodies (mAbs) recognize their cognate epitope displayed on recombinant φX-L2 particles, enzyme-linked immunosorbent assays (ELISAs) were performed as described in *Materials and methods*. We compared anti-L2 antibody binding to wild-type φX174, recombinant φX-L2, and free $L2_{(13–36)}$ peptide. The free and displayed peptide concentrations were identical. Thus, differences may reflect whether a particular antibody has a preference for free or constrained antigen. To determine whether the global suppressors of G protein folding defects alter antibody recognition, φX-L2 particles containing the A83V, A83D, and A106D substitutions were also examined.

As can be seen in Fig. 2, mAbs RG-1, $K18L2_{(20–38)}$, and $K4L2_{(20–38)}$ efficiently and specifically bound the recombinant φX-L2 particles in all genetic backgrounds. No significant reaction was observed with wild-type φX174. The mAbs appeared to differ in their preference for displayed or free L2 epitope. mAb RG-1 bound more efficiently to φX-L2 particles (Fig. 2A), whereas mAb K4 had a preference for the free peptide (Fig. 2C). By contrast, mAb K18 bound equally well to antigen in either context (Fig. 2B). The presence of the *ts* protein suppressors did not appear to significantly affect mAb binding (Fig. 2A–C). Using commercially available antibodies against the HA, myc, and BTAG epitopes, direct ELISA was performed with the respective φX-HA, φX-myc, and φX-BTAG recombinant particles. Again, the recombinant antigen-displaying particles were specifically recognized, whereas no significant reaction was observed with wild-type φX174 particles (Fig. 2D–F). These data suggest a broad utility for φX174G protein antigen display.

In summary, we have explored bacteriophage φX174 as a peptide display platform and we have identified Thr21 as a permissive site for display from the G protein spikes at the vertices of φX174. Although several specific insertions caused a *ts* defect in G protein folding, suppressor mutants of the *ts* phenotype were readily isolated. In all cases tested, the inserted epitopes were efficiently displayed and readily bound by specific antibodies. The apparent plasticity of the Thr21 site, tolerating 13 of 16 insertions, make φX174 an attractive alternative for a variety of peptide and antigen display applications. As with other bacteriophages, large quantities of mature and empty particles (DNA-less) can easily be generated and purified, facilitating their use in such applications (Cherwa et al., 2011; Dokland et al., 1997; Roof et al., 1994). Interestingly, there is a historical precedent of using infectious φX174 particles to study antibody responses and immunoglobulin class switching in animals and humans (Mc Guire et al., 2010; Ochs et al., 1971, 1986; Peacock et al., 1973; Smith et al., 2014), a salient feature which may offer commercial and regulatory advantages should φX174-based vaccine technology be further developed.

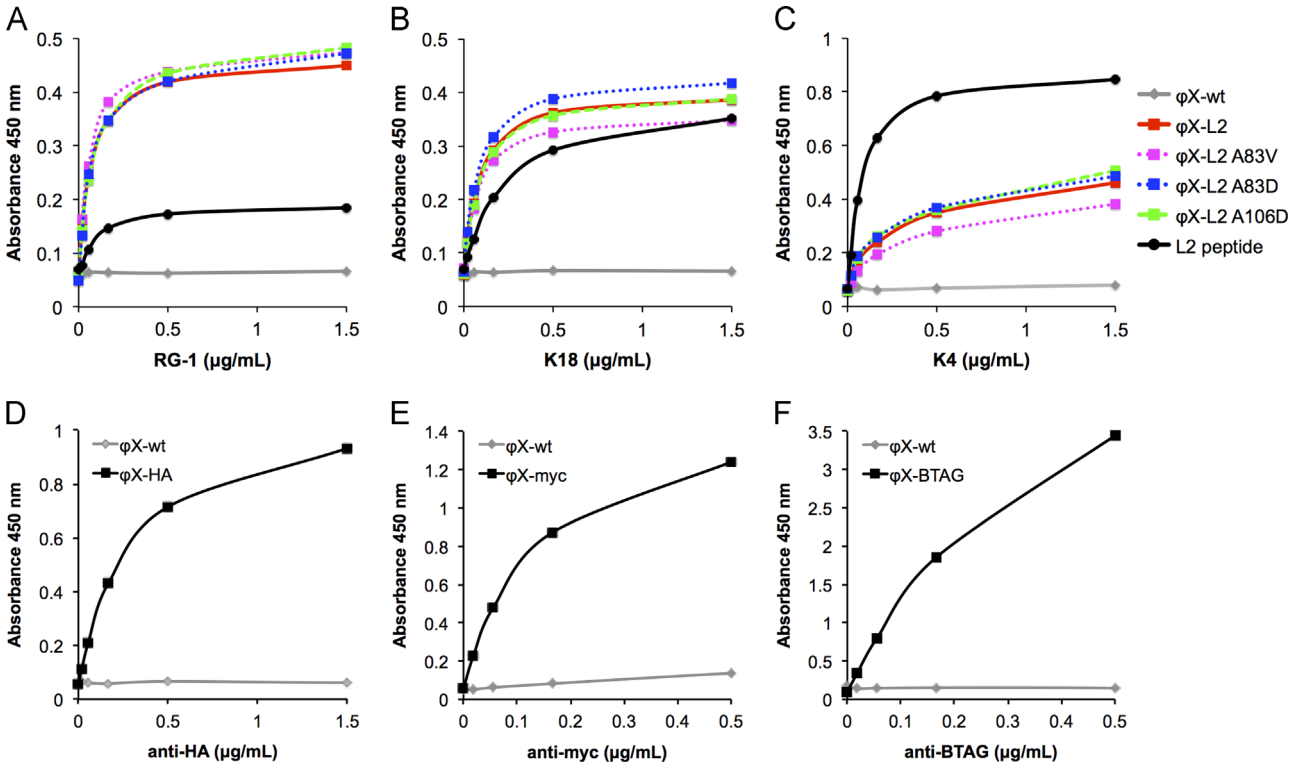

Fig. 2. : Antigen display on the surface of recombinant φX174 particles. (A, B, C) Detection of L2 antigen by direct ELISA of free $L2_{(13–36)}$ peptide, wild-type φX174, φX-L2, and φX-L2 suppressor mutant particles with (A) RG-1, (B) K18, and (C) K4 anti-L2 monoclonal antibodies. Equimolar amounts of L2 antigen, 100 ng of φX174 particles or 6.26 ng $L2_{(13–36)}$ peptide, were pre-bound to each well. Direct ELISA comparing wild-type φX174 to (D) φX-HA with anti-HA antibody, (E) φX-myc with anti-myc antibody, and (F) φX-BTAG with anti-BTAG antibody.

Materials and methods

Phage plating, media, buffers, stock preparation, kinetic assays, and particle synthesis

The reagents, media, buffers, and protocols have been previously described (Fane and Hayashi, 1991). To assay phage stability, 10^8 plaque-forming units (pfu) were added to 10 ml of prewarmed media (1.0% tryptone, 0.5% KCl, 0.01 M $MgCl_2$, 0.005 M $CaCl_2$). Aliquots were removed at regular time intervals, placed on ice, and surviving phages quantified by standard plating assays. Protocols for attachment and eclipse assays (Cherwa and Fane, 2009), and particle purification by rate zonal sedimentation (Cherwa et al., 2011) have been previously described.

Bacterial strains, phage strains, and plasmids

The wild-type *Escherichia coli* C strain C122 (*sup*°), amber suppressing strain BAF5 (*supE*) and strain BAF30 (*recA*) have been previously described (Fane and Hayashi, 1991; Fane et al., 1992). The host slyD mutation confers resistance to E-protein-mediated lysis (Roof et al., 1994).

To clone the wild-type G gene with 5′ and 3′ regulatory regions, nucleotides 2360-3150 from the φX174 genome were amplified by PCR. The upstream primer introduced an Nco I site. The resulting fragment was digested with Nco I and Hae III. The Hae III site is located at nucleotide 3128. It was inserted into the IPTG-inducible bacterial expression plasmid pSE420 (Invitrogen) between the Nco I and Stu I sites. The plasmids pφXG21-$L2_{(20-29)}$, pφXG47-$L2_{(20-29)}$, pφXG151-$L2_{(20-29)}$, pφXG153-$L2_{(20-29)}$ were generated by overlap extension PCR of the G gene, and cloned into pSE420 with Nco I and Hind III, which is located downstream from the Stu I site. The $L2_{(20-29)}$ insertion was engineered to have flanking *Bam*HI and *Bsp*EI sites, which encode Gly-Ser and Ser-Gly linkers. Plasmids pφXG21-myc, pφXG21-HA, pφXG21-$L2_{(16-36)}$, pφXG21-BTAG, pφXG21-PSTCD, pφXG21-M2a, pφXG21-M2b, pφXG21-M2c, pφXG21-CSa, pφXG21-CSc, pφXG21-CSd, and pφXG21-CSe were generated from pφXG21-$L2_{(20-29)}$ by *Bam*HI/*Bsp*EI replacement of the $L2_{(20-29)}$ insert with new inserts generated by either annealed oligonucleotides or PCR.

Complementation and recombination rescue assays

To assay for recombinant G protein complementation, *am(G)Q*33 was plated on a plasmid containing cell line with ampicillin (25 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations between of 0.5–5 mM, and incubated for 4–24 h at 33 °C. IPTG-mediated induction was either toxic to cell viability, yielded plaques, or did not complement with *am(G)Q*33. To rescue viable recombinants, *am(G)Q*33 was plated on a given cell line with ampicillin but without IPTG, and incubated for 6 hours at 33 °C. Resulting plaques were stabbed into C122 (*sup*°) lawns incubated at 33° and 42°. Putative recombinant phages were identified by altered growth patterns and verified by direct DNA sequence analyses.

Isolation of second site suppressor of temperature-sensitive phenotypes

The recombinant strains φX-L2, φX-M2b,φX-M2c, φX-CSa, and φX-HA served as the starting point for the isolation of second site suppressors. The second site suppressors were selected for their ability to suppress high temperature growth defects at 42 °C. Due to their leaky phenotype, approximately 1000 plaque-forming units of φX-L2, φX-M2c, and φX-CSa were plated at 42 °C and incubated for 4 h on the wild-type host C122. The plate contents were washed with 5.0 ml HFB and wash dilutions were plated. Large plaques exhibiting reversion of the *ts* phenotype were picked and the second site suppressors were identified by a DNA sequence analysis. As the *ts* phenotype of φX-HA and φX-M2b was tight, second site suppressors were directly selected from utilizing 10^6–10^7 pfu.

Phage concentration estimation

Purified phage sample was compared by SDS-PAGE with procapsid marker and 1.6 μg bovine serum albumen (BSA). Intensities of the sample coat F protein and BSA bands were analyzed by densitometry and used to estimate the total phage concentration of the sample. The viral coat protein and BSA have similar molecular weights.

L2 peptide synthesis

Free $L2_{(13-36)}$ peptide antigen (FITC-Ahx-ASATQLYKTCK-QAGTCPPDIIPKV-acid) was obtained from Pepscan (Lelystad, Netherlands) and was synthesized with an N-terminal FITC group and a C-terminal carboxylic acid group by solid phase Fmoc chemistry. Peptide was purified to 95% as determined by analytical HPLC and validated by LC/MS. Peptide was dissolved in deionized H_2O and stored at −80 °C.

Direct ELISAs

Wild-type φX174, recombinant phages or free L2 peptide were diluted into PBS buffer (2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.06 mM Na_2HPO_4-$7H_2O$, pH 7.4) and distributed into a 96-well ELISA microtiter plate (Nunc Medisorp). φX174 phages were bound at 100 ng/well and free L2 peptide was bound at 6.26 ng/well, which corresponds to an equimolar amount of L2 antigen. The plate was shaken at 700 rpm, room temperature, for 1 hour to bind targets to the plate. The plate contents were then removed and blocking solution (TBST, 3% BSA, 1% goat serum) was applied to the bound targets by shaking for 30 min. The plate contents were removed and antibody diluted in TBST was bound by shaking for 1 hour. Mouse anti-L2 antibodies RG-1 (Gambhira et al., 2007), and $K18L2_{20-38}$ (K18), and $K4L2_{20-38}$ (K4) (Rubio et al., 2011) were generous gifts from R. Roden and M Müller, respectively. Mouse anti-HA (Applied Biological Materials #G036), mouse anti-myc (GenScript #A00704), and rabbit anti-BTAG (GenScript #A00638) were used. Mouse sera from immunization experiments was mixed and briefly pelleted at 8,000 × g prior to preparation of serial dilutions in TBST. The plate contents were removed and washed four times with TBST by shaking for several minutes. Secondary antibody (goat anti-mouse-HRP conjugate or anti-rabbit) diluted in TBST was added. The plate contents were removed and washed four times with TBST by shaking for several minutes. TMB substrate (Calbiochem #613544) was added and shaken until the signal was visually strong (2–10 min). The reaction was stopped with 2.0 M HCl, and the absorbance at 450 nm was measured with a DTX-800 multimode plate reader (Beckman Coulter).

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