# Factors limiting display of foreign peptides on the major coat protein of filamentous bacteriophage capsids and a potential role for leader peptidase

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**Abstract Many small peptides can be displayed on every copy of the major coat protein in recombinant filamentous bacteriophages but larger peptides can only be accommodated in hybrid virions mixed with wild-type protein subunits. A peptide insert of 12 residues capable of display at high copy number in a hybrid virion was found to be incapable of supporting recombinant virion assembly, a defect that could not be overcome by over-expressing leader peptidase in the same *Escherichia coli* cell. In contrast, over-expressing leader peptidase did increase the copy number of two 9-residue peptides that were poorly incorporated into hybrid virions. The factors that limit peptide display are varied and not restricted to the early stages of viral assembly.**



*Key words:* Filamentous bacteriophage; Peptide display; Membrane protein translocation; Leader peptidase; Peptide library

## 1. Introduction

The filamentous bacteriophage fd can be engineered to display small (6–8 residue) foreign peptides in the N-terminal region of all 2700 copies of the major coat protein (pVIII) of recombinant virions, but larger peptides can only be accommodated in hybrid virions in which modified and wild-type coat protein subunits are interspersed [1,2]. pVIII contains 50 amino acid residues and is largely α-helical. It is synthesised as a precursor, termed pro-coat, with an N-terminal signal peptide of 23 amino acids. The pro-coat is inserted into the inner membrane of the *Escherichia coli* cell and processed to form mature pVIII by leader peptidase [3,4], independently of the *sec* translocation apparatus [5]. A good correlation has been found between the effect of a peptide insert on the rate of processing of the pro-coat and the number of copies of mature modified protein in the assembled hybrid virion [6]. Defects in either membrane insertion or cleavage of the pro-coat by leader peptidase could lower the processing rate and hence the incorporation of a modified coat protein into hybrid virions.

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Foreign peptides are incorporated into the N-terminal region of pVIII without any detectable effect on the structure of the viral capsid, as judged by X-ray fibre diffraction, and model building suggests that the peptide inserts should be able to adopt folded conformations well clear of the viral surface [6]. The antigenic properties of displayed peptides [7,8], together with direct structural analysis of peptide inserts by means of NMR spectroscopy [9], are consistent with this view. Structural constraints at the surface of the virion are therefore unlikely to limit the size of peptide insert in pVIII.

We have modified the genome of the filamentous bacteriophage fd to include a gene encoding β-lactamase. This enabled us to select ampicillin-resistant *E. coli* cells that harbour recombinant virions with a 12-residue insert in every copy of pVIII, but such virions did not form plaques and were produced in vanishingly small quantities when compared with wild-type bacteriophage. The simultaneous over-expression of leader peptidase from a plasmid in the *E. coli* host cells did not enhance the production of recombinant virions displaying the 12-residue peptide. In contrast, over-expression of leader peptidase did increase the copy number of two 9-residue peptide inserts displayed at low levels in hybrid virions, suggesting that different factors affect display of different peptides at different stages in the virus assembly pathway.

## 2. Materials and methods

### 2.1. Materials

Bacteriological media were from Difco. *E. coli* strain TG1 *recO* (K12,Δ*lac-proAB*), *supE, thi, hsd*Δ5, *recO::*Tn5 Kan$^r$/F′*tra*D36, *proA*$^+$*B*$^+$, *lacI*$^q$1, *lac*ZΔM15) used in all experiments was the gift of Dr. P. Oliver (Department of Genetics, University of Cambridge). The plasmid pRD8 was a gift of Prof. Dr. A. Kuhn (Institut für Mikrobiologie, Universität Hohenheim, Stuttgart). All enzymes were from Pharmacia LKB Biotechnology, unless otherwise stated. Polymerase chain reactions (PCR) were performed with *Pfu* DNA polymerase (Stratagene).

### 2.2. Construction of phage display vectors

Recombinant DNA procedures were carried out as described elsewhere [10]. All DNA sequences were confirmed by automated DNA sequencing. Plasmids pfd8B and plasmid pKD encoding gene *VIII* with the peptides ALWGFFPVL and PKLRGDLQVLAQ, respectively, inserted between amino acids 3 and 4 of the mature pVIII have been described previously [6]. To create the bacteriophage vector fdAMPLAY8, new restriction sites for *Xma*I (a *Sma*I isoschizomer) and *Xba*I were introduced into the intergenic region of wild-type bacteriophage fd by site-directed mutagenesis using the oligonucleotide OLfdXEXrev. (5′-CAAAAATCCTTATAAATCTAGAGAA-TTGCCCGGGTTAGTTGTGCGTG-3′) generating bacteriophage fdSX. The primers OLPKXb1for. (5′-CGCACTCCCGTTCTAGA-TAATGTTTTTTGC-3′) and OLPKXb1rev. (5′-GGGATTTTGG-TCTAGAGATTATCAAAAAGG-3′) were used to amplify a DNA fragment consisting of the *tac* promoter, the gene *VIII* and the


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β-lactamase-encoding gene from the vector pfd8SY [11]. The PCR product was digested with *Xba*I and cloned into *Xba*I-digested fdSX, to generate fdPSY. This bacteriophage vector was digested with *Xma*I and *Hin*dIII and the linker 5′- CCGGGCTATTCTTTTG-3′, 3′-CGATAAGAAAACTCGA-5′ was cloned into it to yield bacteriophage vector fdAMP which lacks a *Hin*dIII site. A *Hin*dIII site was created in the gene *III* of fdAMP by site-directed mutagenesis using the oligonucleotide OLfdH3rev. to yield fdAMPH3 (an N15A mutation was introduced in the mature gene *III* protein). The DNA fragment containing gene *VIII* generated by *Sna*BI/*Hin*dIII digestion of pfd8SY [11] was cloned into the *Sna*BI/*Hin*dIII-digested RF DNA of fdAMPH3 to yield fdAMPLAY8 (Fig. 1). The presence of the gene encoding β-lactamase confers ampicillin resistance on virus-infected cells.

A PCR fragment obtained by using the oligonucleotides OLfdHSNBIfor. (5′-GGAAACAGAATTTACGTATTTTACCCG-3′) and OLfdHSTYIrev. (5′-GGGAGTCAAAGGCCGCCTTGGCGGGATCGTT-3′) as primers and pKD [6] as the template was digested with *Sna*BI/*Sty*I and cloned into similarly digested pfd8SY [11] to generate pfd8D. A PCR product was obtained by using pfd8D as the template and the oligonucleotides OLfdRIfor. (5′-CGTTTTAGGTTGGTGAATTCGTAGTGGCAT-3′) and OLfdH3rev. (5′-CGTTAGTAAATGAAGCTTCTGTATGAGGTT-3′) as primers. This product was digested with *Sna*BI/*Hin*dIII and cloned into fdAMPLAY8 to create fdAMPD. Cloning the *Sna*BI/*Hin*dIII fragment from the plasmid pfd8N [6] into the similarly digested RF DNA of bacteriophage fdAMPLAY8 created the bacteriophage fdAMPN. The plasmids pTfd8SY and pTfd8D were obtained by cloning the *Eco*RI/*Sca*I fragments from plasmids pfd8SY and pfd8D into the plasmid pTfd8SHU [12]. The oligonucleotides IN-1 (5′-GGCATTCTGGGTTTCGTGTTCACGCTG-3′) and IN-2 (5′-CAGCGTGAACACGAAACCCAGAATGCC-3′) were treated with polynucleotide kinase, annealed together and ligated into *Hpa*I-digested pKfdH [1] to form plasmid pKIN encoding the peptide GILGFVFTL in pVIII.

### 2.3. Construction of leader peptidase expression plasmid

The L-ribulokinase promoter (*ara*B) contained in the *Sal*I/*Ava*I fragment from the vector pRD8 [13,14] was first cloned into the plasmid pACYC184 (New England Biolabs) to obtain the plasmid pARA. The *Sal*I/*Sma*I fragment from pRD8 containing the leader peptidase gene (*lep*) and the *Sal*I/*Ava*I fragment from pARA containing the *ara*B promoter were then cloned into *Ava*I/*Eco*RV-digested pACYC184, to yield the plasmid pLEP (Fig. 2).

### 2.4. Purification and characterisation of recombinant and hybrid virions

A single plaque of bacteriophage fd was introduced into 1.5 ml of a 1:100 dilution in 2×TY medium of an overnight culture of *E. coli* cells transformed (if appropriate) with the relevant plasmid(s). The medium for cells expressing the pLEP plasmid also contained 200 μg/ml L-arabinose and 50 μg/ml chloramphenicol. The culture

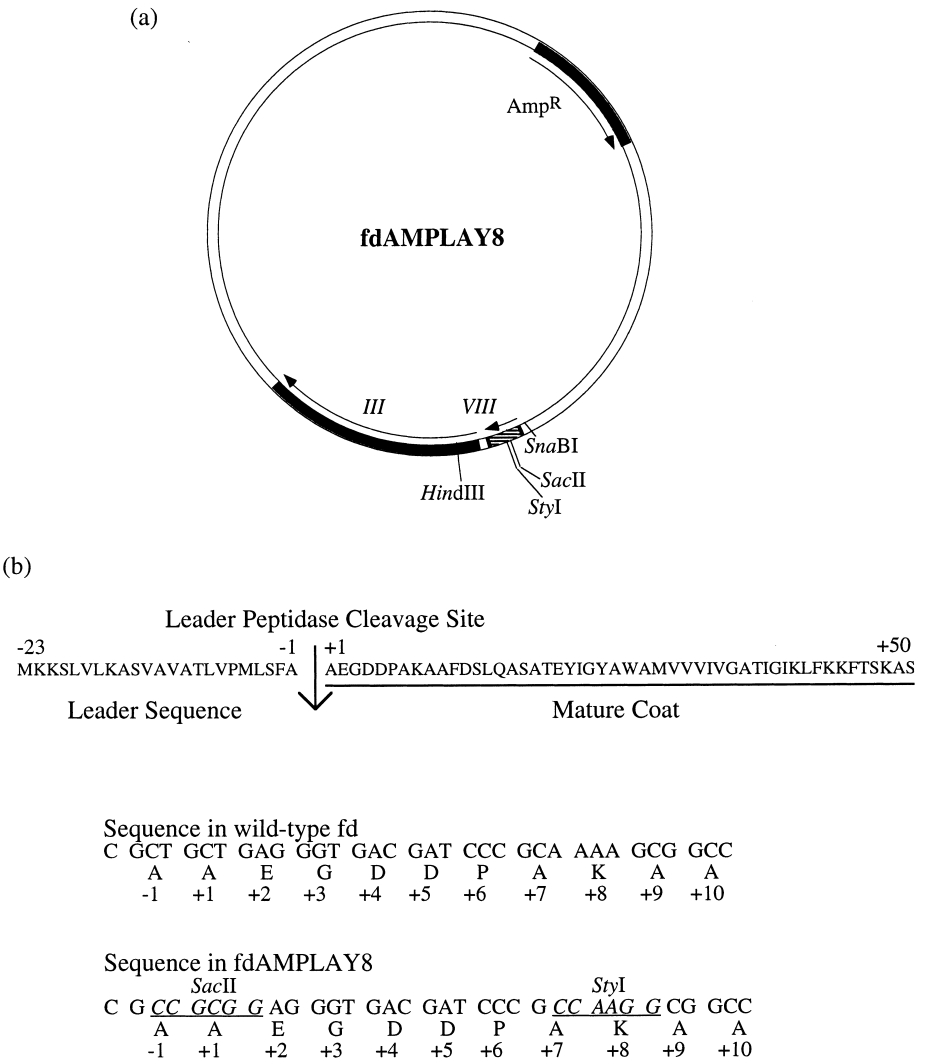


Fig. 1. (a) Schematic diagram of the genome of bacteriophage vector fdAMPLAY8 used for making recombinant bacteriophage virions; Amp$^R$, gene encoding ampicillin resistance; *VIII*, gene *VIII* of bacteriophage fd; *III*, gene *III* of bacteriophage fd. (b) Amino acid sequence of the pro-coat of bacteriophage fd plus the DNA and amino acid sequences corresponding to residues −1 to +10 in wild-type fd and fdAMPLAY8. The cloning sites are underlined and indicated in italics.

was grown at 37°C until turbid and then used to inoculate 100 ml of fresh medium. For induction, IPTG was added (final concentration 10 μM) when the culture reached an $A_{600}$ of 0.9–1.0. The bacteriophage virions were precipitated from the culture supernatant and subjected to N-terminal sequence analysis [6,15].

## 3. Results

### 3.1. Display of long peptides in recombinant virions

Bacteriophage fdAMPLAY8 (Fig. 1) has a gene encoding β-lactamase in the intergenic region, conferring ampicillin resistance on infected cells. The wild-type gene *VIII* of fdAMPLAY8 was replaced with gene *VIII* from plasmid pKD [6] creating bacteriophage fdAMPD with a 12-residue peptide D, PKLRGDLQVLAQ, inserted between amino acids 3 and 4 in the mature pVIII. This peptide was chosen because the corresponding pro-coat is known to undergo rapid membrane insertion and processing by leader peptidase and the mature pVIII is displayed at high levels in hybrid virions [6]. *E. coli* cells transfected with fdAMPD DNA did not form the usual plaques on a lawn but did grow as colonies on a plate made up in 2×TY medium containing 100 μg/ml ampicillin. The DNA from a single colony was sequenced to confirm the presence of the peptide D insert in the bacteriophage pVIII. No virions could be isolated by the usual PEG precipitation of a culture supernatant. After treatment of the culture supernatant at 60°C for 20 min to kill any remaining *E. coli* cells, fresh cells were transduced to ampicillin resistance with a tu of about $10^4$ per ml, a value well below that of bacteriophage fdAMPLAY8 or wild-type fd (both about $10^{11}$ per ml).

As a control, we repeated the experiment with the recombinant bacteriophage fdAMPN, displaying the 6-residue insert VIAMPS, whose pro-coat is also capable of undergoing rapid membrane insertion and processing [6]. Unlike bacteriophage fdAMPD, this recombinant bacteriophage generated plaques on a lawn of *E. coli* cells (although smaller than the plaques generated by bacteriophage fdAMPLAY8) with a tu of about $10^{11}$ per ml.

*E. coli* cells were also transformed with pTfd8D which encodes pVIII containing the same peptide D insert as fdAMPD and additionally confers tetracycline resistance. These cells were infected with bacteriophage fdAMPD and grown in

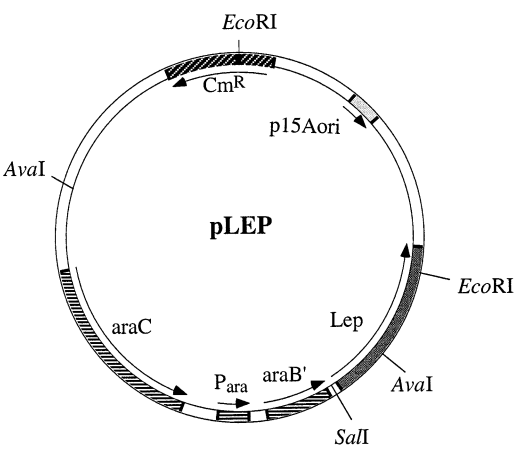


Fig. 2. Schematic diagram of the vector pLEP. The plasmid has the p15A origin of replication and contains the leader peptidase gene (*lep*) under the influence of the *ara*B promoter, $P_{ara}$. It also carries the chloramphenicol resistance marker, $Cm^R$.

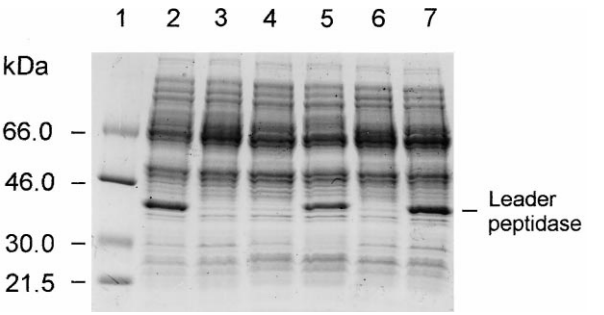


Fig. 3. Over-expression of leader peptidase in *E. coli* cells. Cells transformed with plasmids pfd8B and pLEP or pKIN and pLEP were infected with bacteriophage fdAMPD. The 2×TY medium contained 50 μg/ml chloramphenicol, 200 μg/ml arabinose and 100 μg/ml ampicillin. The cell lysates were submitted to SDS-PAGE. Lane 1, molecular weight markers; lane 2, pLEP; lane 3, pKK223; lane 4, pfd8B; lane 5, pfd8B+pLEP; lane 6, pKIN; lane 7, pKIN+pLEP.

2×TY medium containing ampicillin (100 μg/ml) and tetracycline (50 μg/ml). As before, a tu of only $10^4$ per ml of supernatant was obtained, despite the additional modified pVIII derived from the plasmid gene, indicating that the low yield of recombinant virions was not due to a lack of modified pVIII in the assembly process.

The plasmid pLEP (Fig. 2) carries a chloramphenicol resistance marker and the gene for leader peptidase under the control of the *araB* promoter. *E. coli* cells containing the pLEP plasmid were infected with fdAMPD bacteriophage and grown under chloramphenicol and ampicillin selection in 2×TY medium containing 200 μg/ml arabinose. Over-production of leader peptidase was confirmed by submitting the cell lysate to SDS-PAGE (Fig. 3). The supernatant from fdAMPD/pLEP cultures still exhibited a tu of only $10^4$ per ml. Thus, over-producing leader peptidase in the infected cell also did not increase the production of a recombinant virion displaying peptide D in all 2700 copies of pVIII.

### 3.2. Effect of over-producing leader peptidase on peptide display in hybrid virions

Two 9-residue cytotoxic T-cell peptide epitopes with amino acid sequences ALWGFFPVL and GILGFVFTL were introduced between residues 3 and 4 of mature pVIII, encoded by plasmids pfd8B and pKIN, respectively. These peptides were chosen because their copy number in hybrid virions is very low compared with other peptide inserts [6]. *E. coli* cells harbouring pfd8B or pKIN were simultaneously co-transformed with pLEP. Control cells were transformed only with the plasmid pfd8B or pKIN. Hybrid bacteriophage virions were produced either with or without induction with IPTG (final concentration of 10 μM). Cells transformed with pLEP were simultaneously induced with 200 μg/ml L-arabinose. Hybrid virions were purified and subjected to SDS-PAGE but no clear resolution of the wild-type and modified coat proteins was achieved owing to the nature of the peptide inserts and lack of additional charged amino acids.

N-terminal sequence analysis was used to determine the extent of incorporation of the modified coat protein [6,15]. The percentage of modified coat protein in bacteriophage virions produced from *E. coli* cells not containing the pLEP plasmid and not induced with IPTG was 5–10% for peptide B and 1% (barely detectable) for peptide IN. Paradoxically,

the percentage display of peptide B decreased to 2–5% after induction with IPTG. These cell cultures also showed a substantial fall in $A_{600}$ on induction, although after a few hours the cells started to grow again and the absorbance recovered. In contrast, display of peptide B in hybrid virions produced from cells expressing pfd8B and pLEP increased to 15–20% and this increased further, to 20–25%, when the cells were induced with 10 μM IPTG. The cells also grew well after the induction, unlike those without the pLEP plasmid. A similar increase in copy number from 1 to 5% of pVIII-IN was observed in hybrid virions prepared from cells expressing pLEP as well as pKIN. In this case addition of IPTG did not increase the copy number further.

## 4. Discussion

X-ray diffraction analysis and model-building experiments [6] reveal no structural impediment to the display of large peptides (up to 5 kDa) in pVIII on the surface of a recombinant filamentous bacteriophage. However, we found it impossible to generate a recombinant virion (fdAMPD) displaying a 12-residue peptide (the culture had a tu of only about $10^4$ per ml) despite the ampicillin selection made possible by the fdAMPLAY8 vector. In contrast, bacteriophage fdAMPN displaying a 6-residue insert achieved a tu of about $10^{11}$ per ml, comparable with wild-type fd. Yet the pro-coats for both recombinant virions undergo membrane insertion and processing at similar high rates in the *E. coli* cell [6]. Moreover, the yield of bacteriophage fdAMPD was not increased by simultaneously over-expressing the modified pro-coat from the plasmid pTfd8D or by over-expressing leader peptidase from plasmid pLEP, in the virus-infected cell. Thus peptide inserts in pVIII can affect virion assembly by mechanisms other than slowing down membrane insertion and processing.

Over-production of leader peptidase, however, did increase the frequency of display of two peptides, B (ALWGFFPVL) and IN (GILGFVFTL), in a hybrid virion. Both peptides were initially displayed at very low frequency (5–10% and 1%, respectively) but in both cases over-expression of leader peptidase increased the copy number of the peptides, to 20–25% and 5%, respectively. Leader peptidase may recognise a specific conformation of the pre-protein [16,17]. The presence of certain peptide inserts in the pro-coat may therefore generate an unfavourable secondary or tertiary structure, which lowers the susceptibility to leader peptidase. The fall observed in the copy number of coat proteins displaying peptide B in hybrid virions produced after induction with IPTG in the absence of additional leader peptidase may be due to the accumulation of modified pro-coat in the *E. coli* cell inner membrane undergoing slow processing. In contrast, the same induction of *E. coli* cells expressing pLEP increased the copy number. This would be consistent with the extra leader peptidase helping to release the pro-coat from the membrane for virus assembly, thereby reducing any damage to the cell membrane.

The present results demonstrate that the barrier to display of large peptides in pVIII on recombinant virions is not simply one of membrane insertion and processing of the modified pro-coat but may arise also at a later stage of the assembly process. It has previously been suggested that a proposed membrane channel of bacteriophage pIV molecules through which the virion emerges from the infected *E. coli* cell may be of insufficient diameter to allow egress of a greatly enlarged capsid [18]. It has also been suggested [19] that the modified pVIII molecule may not be able to interact properly with pVII, a minor coat protein found at one end of the assembled virion, to initiate the viral assembly process. Such impediments in the assembly pathway may make it impossible to generate a recombinant virion in which each copy of pVIII displays a peptide of more than about six to eight amino acid residues. The simultaneous provision of wild-type pVIII overcomes this limitation, perhaps by initiating the assembly process and, if necessary, by ensuring that the resultant hybrid virion is still capable of passing through the pIV channel.

Nonetheless, the ability to increase the copy number of peptides displayed at low frequency in hybrid virions by over-producing the *E. coli* leader peptidase significantly extends the utility of the filamentous bacteriophage system for the multiple display of foreign peptides. This could be of importance in various applications, such as the use of the virions in vaccine design.

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