DNA Cloning Vectors Utilizing Replication Functions of the Filamentous Phages of *Escherichia coli*

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**INTRODUCTION**

Two types of prokaryotic cloning vectors can be distinguished: extrachromosomal plasmids, which cause antibiotic resistance of transformed cells, and bacteriophages like phage λ and filamentous phages, which are mostly selected by plaque formation. Among plasmids the most versatile vectors for *Escherichia coli* transformation are those which are derived from the ColE1 plasmid, such as pBR322, pBR325 and pBR328 (Bolivar *et al.*, 1977; Bolivar, 1978). They multiply to more than 50 copies per cell, and are relatively insensitive to the size of the insert. They do not autonomously transfer to other *E. coli* cells, although they can be mobilized by other plasmids like pRK2013 (Ditta *et al.*, 1980). Improvements in cellular transformation methods (Dagert & Ehrlich, 1979; Hanahan, 1983) have increased the efficiency of uptake of DNA for some *E. coli* strains to more than $1 \times 10^8$ transformants per μg DNA. After insertion into the vector DNA a foreign gene can be expressed to such an extent that its product comprises more than 50% of the cellular protein, provided the gene is controlled by a strong promoter like the inducible promoters of phage λ (Remaut *et al.*, 1981), the lac promoter (Fuller, 1982), the trp promoter (Hallewell & Emtage, 1980), the synthetic tac promoter (Amann *et al.*, 1983; De Boer *et al.*, 1983), a phage T7 promoter in the presence of T7 RNA polymerase (Tabor & Richardson, 1985) or by constitutive viral promoters that are dependent on *E. coli* RNA polymerase, together with an efficient terminator (Gentz *et al.*, 1981). The use of inducible promoters or the choice of low copy number plasmids may often circumvent the problem of interference by products from cloned genes with host cell metabolism.

Random insertion of restriction fragments into the DNA of plasmids or filamentous bacteriophages selects for small inserts, because ligation is more readily achieved for small inserts, and the uptake of small plasmids is favoured by the transformation process. Re-ligation of the vector can be inhibited by treatment of cleaved vector DNA with alkaline phosphatase (as a manual for many cloning techniques, see Maniatis *et al.*, 1982). Successful insertion of DNA can be screened for by the loss of antibiotic resistance, or by hybridization of transformed cells on plates with a probe containing part of the insert or a partially homologous sequence. Another approach is based on the complementation of defective cellular β-galactosidase by a DNA segment carrying the lac promoter and a polylinker in phase with the early coding region of β-galactosidase (Messing, 1983). This method, originally developed for plaque screening of filamentous bacteriophage M13, is also useful for plasmids (Vieira & Messing, 1982) and will be discussed later.

The preferential insertion of small DNA fragments can be circumvented by using certain phage λ derivatives (Karn *et al.*, 1983) or by cloning into cosmids and packaging the hybrid DNA *in vitro*. After infection the λ phages are propagated lytically or in the lysogenic state with the host genome. For lysogens a heat-labile λ cI promoter allows induction of the lytic cycle and can also induce expression of a cloned gene via derepression of the λ P$_R$ or P$_L$ promoter after temperature shift. DNA insertions from 70% to 110% of the λ genome (48 514 bp; Sanger *et al.*, 1982) can be tolerated. Phage λ packaging can also be applied to cosmids, which combine plasmid pBR322 with the phage λ-packaging sequence (Collins & Hohn, 1978; Hohn, 1979).
These cloning vectors select for large inserts by packaging into phage λ heads. The phage-like particles with cosmid DNA infect cells like λ does, but once inside the cell the DNA is propagated under the control of the pBR replication origin. The packaging efficiency for cosmids is generally lower than that for phage λ DNA, and the tendency of cosmids to self-ligate requires further screening for DNA insertions.

For many purposes in molecular biology, e.g. nucleotide sequencing by the chain termination method (Sanger et al., 1977, 1980) or for hybridization to RNA, vectors which produce single-stranded DNA are advantageous. For E. coli, two phage types packaging circular single-stranded DNA have been well investigated. The first class are the icosahedral phages exemplified by bacteriophage φX174. The use of this phage as a cloning vector is limited due to its narrow size range and its strict upper limit in DNA packaging. A mixed pBR–φX174-ori vector has been constructed and packaged after superinfection with a φX174 helper phage (Van der Ende et al., 1982). A widely used phage type for packaging DNA in the single-stranded form is filamentous bacteriophage. This type of phage can adjust its coat to accommodate a wide range of DNA, allowing insertions up to 15 kb, although with an increasing tendency for deletions as a consequence of large DNA insertions. Many useful constructions exploiting these phages as cloning vectors have been described (Messing, 1983; Zinder & Boeke, 1982). After infection the viral single-stranded DNA is converted into the double-stranded replicative form, which can be easily isolated from infected cells and used for insertion of foreign DNA. After transformation of competent cells the double-stranded phage DNA continues the viral life-cycle, finally entering the stage of single strand synthesis and phage production. The competence of E. coli cells for double-stranded DNA is usually high after their treatment with Ca²⁺ ions. Single-stranded DNA is efficiently transferred into E. coli cells when they have been converted with lysozyme into spheroplasts (Guthrie & Sinsheimer, 1963). An F− strain as recipient for the transfecting DNA and F+ cells as indicator for plaques should be used for filamentous bacteriophage (Uhlmann & Geider, 1977). To discriminate further between the uptake of single strands and double strands, single-stranded DNA can be covered with a single strand DNA-binding protein like E. coli DNA binding protein I (SSB protein). The formation of this complex strongly reduces the uptake of single strands but not that of double-stranded DNA (Uhlmann & Geider, 1977; Hayes & LeClerc, 1983).

Cloning vectors with the replication functions of filamentous bacteriophage which propagate their DNA in a double-stranded state normally using a ColE1 replication origin (Dente et al., 1983; Zagursky & Berman, 1984) have been described. Infection of carrier cells with a helper phage induces packaging of these plasmids. Other plasmid vectors replicate solely in the phage mode (Geider et al., 1985). In this case viral gene 2 protein has to be provided in the plasmid carrier cells.

This review will consider the properties, applications, advantages and limitations of vectors derived from filamentous bacteriophage. The wide use of these cloning systems limits the citations to a few examples. A more extensive coverage may be obtained by a computer-aided search of the recent literature, which is now available via several data banks (Williams, 1985).

The mode of propagation of filamentous bacteriophage

From the numerous isolates of filamentous bacteriophages three have been well characterized in the last 20 years: fd, fl and M13. The genomes of these phages have been sequenced and only minor deviations have been detected (see Van Wezenbeek et al., 1980; Beck & Zink, 1981). They are very similar in their gene functions and molecular mode of propagation (recently reviewed by Zinder & Horiuchi, 1985; Baas, 1985).

The filamentous bacteriophages infect cells via F-pili, although high multiplicities of the phage can also cause rare infections of F− cells (K. Geider, unpublished). The first mature phages appear within 15 min. Phages can also be expected at a similar time after cell transformation with double-stranded DNA. Filamentous bacteriophages are not lytic, they coexist with the infected cells for many generations. If the products of cloned genes cause toxic effects in cells they can be inserted into filamentous bacteriophage and thus brought into cells by phage infection at any growth stage of the cells.
The genome of filamentous bacteriophages has a size of 6408 (fd) or 6407 bp (f1, M13) representing nine genes and an intergenic region (IG) (Fig. 1). A tenth gene is translated from the region of gene 2 coding for the C-terminal end of the protein. Mutation analysis and gene complementation demonstrated the necessity of gene 10 for viral DNA synthesis (Fulford & Model, 1984). Genes 2, 10, 5, 7, 9 and 8 are strongly expressed, increasingly in that order, and gene 8 is followed by a strong terminator that allows some readthrough for the expression of gene 3. Genes 3, 6, 1 and 4 are infrequently expressed. This also occurs in a cascade system which is efficiently terminated by the intergenic region (Smits et al., 1984) located between gene 4 and gene 2. It has a size of 507 nucleotides and neither codes for known peptides nor for RNA or readthrough transcripts. For phage fd the longest possible reading frame solely located within this region comprises less than 200 nucleotides. The intergenic region contains four hairpin-like structures, which are thought to be involved in replication and packaging of the viral genome (Fig. 2). The first major hairpin carries the cleavage site for gene 2 protein (Meyer et al., 1979; Meyer & Geider, 1979), which initiates the synthesis of the viral strand. The second and third hairpins are involved in the start of complementary strand synthesis (Geider et al., 1976). The primer for initiation of the complementary strand is synthesized at the beginning of the second hairpin, but binding of the initiating RNA polymerase (Geider & Kornberg, 1974) also protects the third hairpin (Schaller et al., 1976). Mutations in this region cause a drastic drop in replication efficiency (Cleary & Ray, 1981; Kim et al., 1981; Geider et al., 1985). Initiation of complementary strand synthesis may then occur at other sites on the plasmid. Ray and co-workers (1982) constructed an M13 vector with deletions of sequences from the complementary strand origin. This phage forms small plaques and can be used to select for inserts with an initiation signal for complementary strand synthesis (Nomura et al., 1982; Strathearn et al., 1984). The fourth hairpin has been implicated in packaging (Schaller, 1979; Dotto et al., 1981; Dotto & Zinder, 1983). To obtain phage formation the orientation of the morphogenetic signal, but not its proximity to the viral strand origin, is important.

The infecting viral strand is prepared for DNA replication by an RNA primer of 26 to 30 nucleotides synthesized by the host RNA polymerase (Geider et al., 1976). It is then converted into double-stranded DNA by *E. coli* DNA polymerase III holoenzyme. The primer RNA is removed by RNase H and the 5′-3′ exonuclease activity of DNA polymerase I, the gap is filled by incorporation by the same polymerase, the strand sealed by *E. coli* DNA ligase, and the DNA is then converted into the supercoiled form, RF1, by DNA gyrase (*E. coli* topoisomerase II). This form is used for activation of viral strand replication by the phage gene 2 protein. Viral strands are replicated in the rolling circle mode and circularized via cleavage of the single-stranded tails and strand sealing by gene 2 protein (Meyer & Geider, 1982). Gene 2 of filamentous bacteriophage has been cloned into pBR plasmids (Meyer & Geider, 1981), thus increasing the
level of the protein far beyond that normally found in phage-infected cells. A further increase of gene 2 protein in cells was obtained by expressing the gene under the control of the strong, inducible araB promoter of Salmonella typhimurium (Johnston et al., 1985). Plasmids having the replication origin of phage fd seem to stimulate the expression of cloned gene 2 (Meyer & Geider, 1981). Gene 5 protein prevents initiation of complementary strand synthesis by binding to the single-stranded tails of the rolling circles. At the bacterial membrane these complexes are converted into phage particles which contain the proteins encoded by genes 3, 6, 7, 8 and 9 (Grant & Webster, 1984). Viral gene 1 protein and cellular thioredoxin may interact in the assembly process (Russel & Model, 1985). The hairpin nearest to gene 4 in the intergenic region is positioned opposite the attachment protein (gene 3 protein) and binds to the minor coat proteins encoded by gene 7 and gene 9 (Armstrong et al., 1983). The mature phages are extruded through the cell envelope without cell lysis; membrane adhesion sites of the infected cells seem to be preferred for the extrusion process (Bayer & Bayer, 1986).

If propagated at high multiplicities, filamentous bacteriophages produce high levels of smaller phage particles, called miniphages (Griffith & Kornberg, 1974; Enea & Zinder, 1975). Their genomes contain the whole intergenic region with parts of the surrounding genes 2 and 4 and can also be used as cloning vectors. Sequences from the intergenic region can occur amplified in these miniphages (Enea et al., 1977) and also in intact filamentous bacteriophage (Schaller, 1979). Replication of miniphages depends on the presence of a helper phage in the same cell, because they lack complete genes. Construction of cloning vectors derived from filamentous bacteriophage demands insertion in non-essential regions without inactivation of the genes required for genome propagation. The intergenic region is quite suitable for artificial DNA inserts, although they may interfere with packaging functions or DNA replication. The favoured sites for inserts are therefore between the packaging signal and the complementary strand replication origin (Fig. 2). Other inserts have been located in the first hairpin of the complementary strand origin (Boeke et al., 1979; Barnes & Bevan, 1983) just after the

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**Fig. 2.** The use of the intergenic region of filamentous bacteriophages for the construction of cloning vectors. The approximate sites of insertions are given for phage vectors. For pfd plasmids and pBR-oriri vectors, the borders are indicated. The nucleotide positions correspond to the fd gene 2 protein cleavage site as given in Fig. 1.
termination sequence of the RNA primer (Geider et al., 1976). Inserts in the second complementary strand hairpin (Geider et al., 1985) or deletion of this part (Cleary & Ray, 1981) reduce the copy number of the genome as mentioned above. Insertions into the replication origin of the viral strand or deletion in this region have been employed for detailed analysis of this region. The viral strand origin can be divided into (i) a core sequence of about 40 nucleotides with the gene 2 protein recognition sequence, which seems to coincide with the termination signal, and (ii) a larger area for viral strand initiation (Dotto et al., 1981, 1984). Deletion mutants have defined the core region as being from 12 nucleotides in the 5' direction from the gene 2 protein cleavage site (Dotto et al., 1982) to 29 nucleotides in the 3' direction (Dotto & Zinder, 1984a; Kim & Ray, 1985). An area of about 100 nucleotides adjacent to the core region enhances viral strand initiation. The effects of disruptions in this area can be suppressed by structural changes in viral gene 2 protein (Dotto & Zinder, 1984a; Kim & Ray, 1985) or by higher intracellular levels of the protein (Dotto & Zinder, 1984b); the latter can be caused by mutation in viral gene 5 protein or by changes in the gene 2 mRNA leader. Gene 5 protein from filamentous bacteriophage is not only a repressor of complementary strand synthesis, but also a regulator for the translation of gene 2 (Model et al., 1982). Owing to their single-stranded genome filamentous bacteriophages cannot correct errors in the base sequence, which may occur in the virus particle. On the other hand, the various mechanisms of suppression of gene 2 mutations indicate an ability to overcome distortions in virus propagation.

**DNA cloning with bacteriophage M13 using lac complementation**

Insertions of DNA into the genome of a filamentous bacteriophage result in larger phage particles, which can eventually become fragile and less effective in DNA replication. Early attempts to create restriction sites for insertion into filamentous bacteriophage DNA were carried out with phage M13 (Messing et al., 1977). The introduction of a DNA fragment with an indicator of insertion has rendered M13 a more convenient cloning vector. This was achieved by insertion of a short stretch of the lac operon (Gronenborn & Messing, 1978). The insert, located on an HaeII fragment, comprises an early segment of the lacZ gene with the lac promoter, operator and repressor. The segment of the lacZ gene produces a protein fragment, the α-protein, which is able to complement a defect at the N terminus of β-galactosidase. To obtain complementation with such an M13 vector the E. coli strain must have an appropriate mutation in the lacZ gene, for instance the ΔM15 deletion. Complementation is visualized as blue plaques on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for colour production and isopropyl-β-D-thiogalactopyranoside (IPTG) for gene induction (Messing, 1983). Insertion of foreign DNA into the lacZ gene, which carries a polylinker region, disrupts the coding sequence for the α-protein. Consequently these phages give rise to white plaques. Phages without insertions or some ‘in frame’ additions retain the blue plaque phenotype. The blue plaque phenotype for the latter insertions can be obtained if the DNA fragment provides a ribosome binding site and an initiation codon distal to the lac promoter of the lacZ' segment (Close et al., 1983). Development of the M13 cloning system has created phages with numerous unique cloning sites (Norrander et al., 1983; Yanisch-Perron et al., 1985). Other sites can be used after partial DNA digestion. ‘In frame’ insertions have been expressed as inducible fusion proteins with the N terminus of the α-protein and a modified human interferon α gene (Fuke et al., 1984) or parts of the α-protein with an adapter sequence joined to the synthetic pre-sequence for human proinsulin (Georges et al., 1984) or with α-protein and interferon α2 (Slocombe et al., 1982). Further, the vector M13mp7 has been changed in such a way that in three versions the HindIII site can be used to translate an insert in phase with the N terminus of β-galactosidase (Lathe et al., 1984).

Visualization of lac complementation can only be achieved when the host cell has a suitable genetic background. The first host strains and M13 vectors were designed on the basis of biological safety requirements; indeed suppressor functions of the bacterium can occasionally be desirable for restriction of the propagation of the viral genome to those cells. But since survival of artificially created potential pathogens and their growth potential outside laboratory conditions seem to be very low, this restrictive genetic background is no longer required,
Table 1. Examples of host strains used for propagation of filamentous phage vectors*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM83</td>
<td>ara, Δ(lac-proAB), strA, φ80d lacZΔM15</td>
<td>1</td>
</tr>
<tr>
<td>JM101</td>
<td>supE, Δ(lac-proAB), thi, [F', traD36, proAB, lacPΔZΔM15]</td>
<td>1</td>
</tr>
<tr>
<td>JM107</td>
<td>hsdR17, supE, Δ(lac-proAB), endA1, gyrA96, relA1, thi, [F', traD36, proAB, lacPZΔM15]</td>
<td>1</td>
</tr>
<tr>
<td>JM109</td>
<td>recA1, hsdR17, supE, Δ(lac-proAB), endA1, gyrA96, relA1, thi, [F', traD36, proAB, lacPZΔM15]</td>
<td>1</td>
</tr>
<tr>
<td>JM110</td>
<td>dam, dem, rpsL, thr, leu, supE44, Δ(lac-proAB), lacY, galK, relA1, galT, ara, tonA, tss, thi, [F', traD36, proAB, lacPZΔM15]</td>
<td>1</td>
</tr>
<tr>
<td>BHB2600</td>
<td>supE, supF lysogenic for λ CH616‡</td>
<td>2</td>
</tr>
<tr>
<td>1101</td>
<td>F+ supE</td>
<td>3</td>
</tr>
<tr>
<td>KB35</td>
<td>F+ supE</td>
<td>4</td>
</tr>
</tbody>
</table>

* Strains 1101 and KB35 (F donors) can be used directly for the transfer of the F episome by conjugation with F− strains (recipient); the JM strains require complementation of the traD gene for F transfer. To verify the F+ character, cells from single colonies are plated to a lawn and droplets of male-specific DNA phages (fd, fl, M13) or RNA phages (fr, f2, R17, MS2) are applied to the surface, or they are infected in culture with a filamentous phage carrying an antibiotic resistance gene for 30 min and then plated on selective agar. The JM strains can also be screened on minimal plates.


‡ λ NM616 with an insert of fd gene 2.

although one should be aware of the existence of mutant phages depending on host suppressors. On the other hand, M13mp8–his phages defective in gene 2 were used to introduce promoter mutations into a deficient his operon of an F+ S. typhimurium strain that did not suppress the viral amber mutation (Artz et al., 1983). A further improvement of E. coli hosts for M13 phages was the transfer of the gene with the lacZΔ deletion to an F episome together with the proAB genes and with the lacI gene which constitutively expresses the lac repressor. To prevent recombination with the lac region of the chromosome, this chromosomal area (lac-proAB) was deleted. The presence of the F episome can be detected by screening for proline prototrophy on minimal agar plates. Furthermore, this specific F+ episome cannot be spontaneously transferred to other strains because of its traD mutation. Strain JM101 has the pro–lac deletion and a suppressor for amber mutations (Table 1). An additional feature of strain JM107 is the hspR17 gene, which produces a deficient host restriction endonuclease (rK12−) without affecting the corresponding methylase (mK12+). This genetic background is important for cloning DNA which is not derived from E. coli K12 strains, because the host-specific restriction system would otherwise degrade 'foreign' inserts. To avoid spontaneous deletions of DNA, particularly of inserts with repetitive sequences, the recA mutation was introduced (JM109). Since cleavage by some restriction enzymes is abolished by the methylation of certain bases, genes encoding methylases (dam, dcm) were mutated in another strain (JM110). There are also strains such as JM83 carrying the lacZΔM15 deletion in the chromosome in a lysogenic defective φ80 phage (Table 1).

Filamentous bacteriophages fd and fl as cloning vectors

Other filamentous bacteriophages like fd or fl can also be used as cloning vectors (Table 2a, b). Indicator functions for phage fd vectors were mainly based on insertion of genes from pACYC- or pBR-based plasmids such as those for kanamycin, ampicillin or chloramphenicol resistance. These genes were mostly inserted into the double HaeII site of the intergenic region at positions 6187/6195 (for numbering see Fig. 1), or a HaeIII site was converted into an EcoRI site without the introduction of a resistance gene (Herrmann et al., 1980). Double resistance allows inactivation of one gene and screening for the intact one. There are also fl phages with artificial EcoRI sites (Boeke et al., 1979; Boeke, 1981) or with ampicillin–tetracycline double resistance (Boeke et al., 1979). After transformation of competent cells some time should be allowed (15 to 30 min) for the phage to establish in the cell before plating on selective agar. Longer incubation will result in the onset of phage production. Expansion of the genome by inclusion of DNA
### Table 2. A selective list of cloning vectors derived from filamentous bacteriophages

#### (a) Phage vectors with selective markers for DNA insertions

<table>
<thead>
<tr>
<th>Vector name</th>
<th>Markers with multiple cloning site</th>
<th>Detection of insertion</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mpl to 19</td>
<td><em>lac</em> complementation</td>
<td>Colour of plaques</td>
<td>1</td>
</tr>
<tr>
<td>M13 bla cat</td>
<td>Antibiotic resistances</td>
<td>Replica plating on selective agar</td>
<td>2</td>
</tr>
<tr>
<td>fd101, 103, 106</td>
<td>Antibiotic resistances</td>
<td>Replica plating on selective agar</td>
<td>3</td>
</tr>
</tbody>
</table>

#### (b) Phage vectors with cloning sites outside marker genes

<table>
<thead>
<tr>
<th>Vector name</th>
<th>Cloning sites</th>
<th>Recognition of transformed cells</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R199, 209</td>
<td><em>EcoRI</em></td>
<td>Plaques</td>
<td>4</td>
</tr>
<tr>
<td>R229</td>
<td><em>EcoRI</em></td>
<td>Plaques</td>
<td>5</td>
</tr>
<tr>
<td>fd11</td>
<td><em>EcoRI</em></td>
<td>Plaques</td>
<td>3</td>
</tr>
<tr>
<td>fd107, 109</td>
<td><em>EcoRI, HindIII, SalI/EcoRI</em></td>
<td>Colonies</td>
<td>3</td>
</tr>
<tr>
<td>mWB2344</td>
<td><em>BglII, EcoRI, HindIII, PstI, PucI, PvuI, PwuI, XbaI</em></td>
<td>Plaques</td>
<td>6</td>
</tr>
<tr>
<td>M13Goril</td>
<td><em>EcoRI, KpnI, PwuI, SsrI, XhoI</em></td>
<td>Plaques</td>
<td>2</td>
</tr>
</tbody>
</table>

#### (c) Plasmid vectors used without amplification as phage particles

<table>
<thead>
<tr>
<th>Vector name</th>
<th>Mode of replication</th>
<th>Remarks‡</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfdA2†, A3†, A4†</td>
<td><em>fd ori, fd gene 2</em></td>
<td>Km, Km/Ap, Km/Cm</td>
<td>7</td>
</tr>
<tr>
<td>pfdA8†</td>
<td><em>fd ori, fd gene 2</em></td>
<td>Km, polylinker</td>
<td>7</td>
</tr>
<tr>
<td>pfdC1†</td>
<td><em>fd ori, fd gene 2</em></td>
<td>Km, autonomous growth</td>
<td>8</td>
</tr>
<tr>
<td>pfdB2</td>
<td><em>fd ori, fd gene 2</em></td>
<td>Km, site for cloning: <em>EcoRI</em></td>
<td>7</td>
</tr>
<tr>
<td>pEMBL8, 9</td>
<td>ColE1 (pBR322)</td>
<td>Polylinker, Ap</td>
<td>9</td>
</tr>
<tr>
<td>pBS8, 9+/-</td>
<td>ColE1 (pBR322)</td>
<td>Polylinker, Km</td>
<td>10</td>
</tr>
<tr>
<td>pEMBL6</td>
<td>ColE1 (pBR322)</td>
<td><em>E. coli</em>-yeast shuttle vector, polylinker</td>
<td>11</td>
</tr>
<tr>
<td>pGX3804, 3805</td>
<td>ColE1 (pBR322)</td>
<td><em>E. coli</em>-B. subtilis shuttle vector</td>
<td>12</td>
</tr>
<tr>
<td>pEMBLex2</td>
<td>ColE1 (pBR322)</td>
<td><em>λ</em> Pr, RBS(MS2 pol)</td>
<td>13</td>
</tr>
<tr>
<td>pEMBLex3</td>
<td>ColE1 (pBR322)</td>
<td><em>λ</em> Pr, cIs857, RBS(MS2 pol)</td>
<td>13</td>
</tr>
<tr>
<td>pSP65ss</td>
<td>ColE1 (pBR322)</td>
<td><em>Pvps</em></td>
<td>13</td>
</tr>
<tr>
<td>pSP64/65-f1+/-</td>
<td>ColE1 (pBR322)</td>
<td><em>Pvps</em></td>
<td>14</td>
</tr>
<tr>
<td>pFEC4</td>
<td>ColE1 (pBR322)</td>
<td>With <em>λ</em> P1 promoter</td>
<td>15</td>
</tr>
<tr>
<td>pKUN9</td>
<td>ColE1 (pBR322)</td>
<td>(+) and (−) strand packaging</td>
<td>16</td>
</tr>
<tr>
<td>pSS24/25</td>
<td>ColE1 (pBR322)</td>
<td>cDNA cloning</td>
<td>17</td>
</tr>
</tbody>
</table>

* References: 1, Messing (1983); 2, Hines & Ray (1980); 3, Herrmann et al., 1980; 4, Boeke et al. (1979); 5, Boeke (1981); 6, Barnes & Bevan (1983); 7, Geider et al. (1985); 8, R. Baldes & K. Geider, unpublished; 9, Dente et al. (1983); 10, Spratt et al. (1986); 11, Baldari & Cesareni (1985); 12, Schmidt et al. (1986); 13, Salazzo et al. (1985); 14, Mead et al. (1985); 15, Lorenzetti et al. (1985); 16, Peeters et al. (1986); 17, Kowalski et al. (1985).
† Vectors without packaging signal. The other vectors can be packaged after infection of carrier cells by a helper phage. The yield is increased by using a natural (Dott & Horiuchi, 1981) or an artificially created (Vieira & Messing, 1986) interference resistant helper phage.
‡ Abbreviations: Km, Ap, Cu, resistance to kanamycin, ampicillin or chloramphenicol; RBS, ribosome binding site; pol, polymerase.

Fragments conferring antibiotic resistance is disadvantageous for further DNA insertion, because very large genomes have a tendency to undergo spontaneous deletion, although some vector constructs appear to propagate large DNA inserts stably in certain strains (Barnes & Bevan, 1983). The rolling circle mode of replication may be unfavourable for transposon insertion, as Tn5 has been found to be rather labile in the fd genome (Herrmann et al., 1978).

### Plasmids using fd replication functions

Less than 250 nucleotides constitute the replication signals for filamentous bacteriophage. A DNA fragment of 260 nucleotides was dissected from the fd genome and linked to a gene for resistance to kanamycin (NPT I) (Meyer & Geider, 1981). Because propagation of this artificial plasmid (pfd) depends on the presence of gene 2 protein in the same cell, the gene coding for this protein was inserted into a pBR plasmid and provided a sufficient supply of the viral replication protein. The helper plasmid could be selectively removed, by cleavage with certain restriction enzymes, from the pfd plasmid which could then be used as a cloning vector.
To avoid the two-plasmid cloning system, a DNA fragment with fd gene 2 was ligated into phage λ which was subsequently inserted into the host chromosome (Geider et al., 1985). Phage fd gene 2 was therefore propagated with the host chromosome, and the pfd plasmid was supplied with sufficient gene 2 protein to allow propagation at copy numbers of about 100 per cell. The pfd plasmids have also been constructed with various double resistances and unique restriction sites. A version with a polylinker outside a resistance gene is also available. These plasmids are neither autonomously transferred nor can they be replicated in the absence of gene 2 protein. They are not homologous to phage λ DNA nor to many pBR plasmids and therefore are suitable as vectors for subcloning DNA with subsequent DNA hybridization. The heat lability of gene 2 protein allows removal of the pfd vectors from cells in the absence of selective pressure. The vectors can also be used therefore for transient labelling of cells with resistance markers or for transfer of a transposon followed by removal of the donor plasmid. The pfdA plasmids (Table 2) cannot be packaged upon infection with a helper phage. Like other plasmids they can be used as vectors for DNA sequencing by chain termination methods, by applying labelled primers to denatured supercoiled DNA. Derivatives of these plasmids can be used for α-complementation; others carry fd gene 2 (pfd C1) thus creating an autonomously replicating pfd plasmid (R. Baldes & K. Geider, unpublished). Much larger inserts can be propagated in these plasmids than can be propagated with intact filamentous bacteriophage. The instability of DNA inserted in filamentous bacteriophage may therefore be due to phage formation and re-infection and not particularly due to the mode of replication. On the other hand, insertion of the phage M13 genome into plasmids causes deletions with the gene 2 protein cleavage site as the predominant endpoint (Michel & Ehrlich, 1986). Therefore, the nick created by gene 2 proteins might be an active site for illegitimate recombination.

Packaging of defective filamentous bacteriophage

The packaging signal of bacteriophage fd is located in a hairpin structure in the intergenic region near gene 4 (Schaller, 1979; Dotto et al., 1981; Dotto & Zinder, 1983). Insertion of sequences with this signal into a pfdA plasmid (pfd B2; Table 2) allows ssDNA of the vector to be packaged in the presence of a helper phage (Geider et al., 1985). Spontaneously derived miniphages with antibiotic resistances can also be used as cloning and packaging vectors in cells lysogenic for a λ phage carrying fd gene 2 (K. Beck, R. Herrmann & K. Geider, unpublished). Successful infection of cells by helper phage can be selected via drug resistance (Geider et al., 1985). Miniphages do not rely only on the presence of the helper phage, but also interfere with the propagation of the helper. In the barely visible plaques interference-resistant phages arise, and produce a normal plaque morphology (Dotto & Horiuchi, 1981). Studies with a complete and with a partially deleted viral replication origin (Zagursky & Berman, 1984) suggest competition for gene 2 protein in the amplification of each genome.

Mixed plasmids with the ColE1 replication origin and part of the intergenic region from filamentous bacteriophage including the packaging signal have been described (Dente et al., 1983; Levinson et al., 1984; Zagursky & Berman, 1984; Peeters et al., 1986; see Table 2 c). They can stably propagate long inserts of DNA due to their ColE1 replication mode, and they are efficiently packaged upon infection with an interference-resistant helper phage. This type of f1-ori-pBR plasmid has been further developed and shuttle vectors (pEMBLY) for cloning and packaging in E. coli and subsequent transformation of yeast cells have been constructed (Baldari & Cesareni, 1985). Other vectors express cloned DNA via phage SP6 promoters (Mead et al., 1985; Sollazzo et al., 1985) or phage λ promoters (Lorenzetti et al., 1985; Sollazzo et al., 1985), or they can be used for cDNA cloning by the Okayama–Berg procedure (Kowalski et al., 1985). Packaging of both strands of an insert can be done with vectors carrying parts of the intergenic region of phage M13 and of phage Ike (Peeters et al., 1986). The latter is a filamentous bacteriophage which infects E. coli cells carrying plasmids of the I and N incompatibility group and whose gene 2 protein and packaging proteins recognize only its own intergenic region, and not that of phage M13. Joint cloning of the intergenic region fragments of each phage in opposite orientations on a pBR plasmid and superinfection with one phage activate only the corresponding packaging signal.
Nucleotide sequencing of DNA insertions

DNA from filamentous phage vectors can be readily isolated in the single-stranded form after purification of the phage thus providing an excellent tool for chain termination sequencing. Sequencing of single-stranded DNA by chain termination methods (Sanger et al., 1980) is at present limited to a length of about 600 bases following the priming oligonucleotide which usually requires subcloning of a long insert in fragments (examples in: Skinner & Siddell, 1983; Etzerodt et al., 1984; Van Wezenbeek et al., 1983). In early applications of this method, mutations in a short segment cloned in M13 derivatives were analysed with a single primer (Demopoulos et al., 1982; Everett & Chambon, 1982). In the case of large DNA segments subcloning of many defined short fragments can be a tedious task. Messing and collaborators (Messing et al., 1981; Messing, 1983) have therefore proposed shotgun cloning and subsequent computerized evaluation of the sequence data (examples in: Gardner et al., 1981; Messing et al., 1984). For confirmation of the sequence data sequencing of strands inserted in both directions is often advisable. After dideoxynucleotide sequencing the more time-consuming method of Maxam & Gilbert (1980) might clarify ambiguous results (example in: Geraghty et al., 1981). Recent improvements like the solid-phase degradation of labelled fragments (Rosenthal et al., 1985, 1986) or the single-step piperidine cleavage procedure (Ambrose & Pless, 1985) may facilitate sequencing according to Maxam and Gilbert. For chain termination sequencing not only the quality of oligonucleotide primers but also intrinsic features of the DNA to be sequenced, especially hairpin structures, can influence the accuracy of the results. Primers available from commercial sources are mostly of good quality as are primers made by automatic synthesizers after purification on polyacrylamide gels or by high-performance liquid chromatography (HPLC). Problems can arise from non-highly standardized laboratory procedures using less efficient coupling steps. The use of unpurified primers (Sanchez-Pescador & Urdea, 1984) or crude DNA preparations will diminish the extent and the accuracy of chain termination sequencing. The attachment of differently coloured fluorescent dyes to the oligonucleotide primers has allowed automated DNA sequence analysis (Smith et al., 1986).

In order to sequence inserts larger than a few hundred nucleotides several approaches have been discussed (Deininger, 1983). Without changing the insert, a second primer initiating the complementary strand after extension of the first one can be used (Hong, 1981). When one segment of the sequence has been determined, another primer starting at the end of the newly determined sequence may be synthesized. Using a sequential set of primers of this kind one can traverse the DNA, provided a supply of newly synthesized oligonucleotides is readily available. For sequencing with a universal primer a series of controlled deletions was introduced into inserts with nuclease BAL-31 (Poncz et al., 1982; Misra, 1985), with exonuclease III (Henikoff, 1984) or by using DNase I together with these two enzymes (Barnes & Bevan, 1983). Primers for chain termination sequencing can also be used for sequencing inserts in the double-stranded DNA of plasmids after linearization and denaturation (Wallace et al., 1981) or after denaturation only, if the plasmids are supercoiled (Chen & Seeburg, 1985). Direct cloning into vectors derived from filamentous bacteriophages allows immediate sequence analysis, as was used in the analysis of the VH regions of immunoglobulin genes (Blankenstein et al., 1984).

Mutagenesis of DNA

Besides the use of single-stranded DNA of filamentous bacteriophage in DNA sequencing by the primer-based chain termination method, the phage genomes can be applied to various other purposes requiring single-stranded DNA. These are hybridization to RNA, chemical mutagenesis of short DNA fragments and the use of synthetic oligodeoxynucleotides in genetic engineering. Only a few examples of the numerous reports in the literature will be cited here.

In order to mutagenize DNA fragments specifically, base changes can be designed by synthesis of oligonucleotides including the appropriate region and annealed to the single-stranded DNA of filamentous bacteriophages (Zoller & Smith, 1983). The efficiency of introducing a mutation is increased if the oligonucleotide is extended by the large fragment of DNA polymerase I, and the double-stranded DNA is converted into a supercoil and then purified in an alkaline gradient. This purification step is not necessary if a second primer is
simultaneously annealed and both primers are used for complementary strand synthesis (Norris et al., 1983; Zoller & Smith, 1984), or if the mutating oligonucleotide is inserted into a gapped duplex DNA (Kramer et al., 1984). To select for the recombination of the annealed oligonucleotide the viral strand may carry amber mutations (Bauer et al., 1985) or an EcoK12 (EcoB) cleavage site (Carter et al., 1985). The mutating oligonucleotide is annealed to the site of interest, and the second oligonucleotide without the amber mutation or with a methylated EcoK12/B site is annealed to the corresponding region of the viral strand. The heteroduplex can then give rise to the production of more than 70% mutant phages among the progeny, even without previous purification of the heteroduplex DNA. The EcoK12 restriction/modification system has also been applied to select for deletions or insertions within its 13-nucleotide recognition sequence (Waye et al., 1985). In order to change a single amino acid, oligonucleotides have been designed to introduce unique restriction sites up- and downstream of the coding sequence without changing other amino acids. Another synthetic fragment was then inserted between the unique cuts which introduced the mutation and removed the previously created restriction sites (Wells et al., 1985). Another approach is the synthesis of a whole DNA segment using overlapping oligonucleotides, which can be individually changed and then inserted together into the polylinker of an M13 vector (Grundström et al., 1985).

To obtain deletions of nucleotides, DNA cloned in a phage M13 vector was annealed to synthetic oligonucleotides which lacked the sequence to be removed from the template DNA. After conversion to dsDNA and cell transformation with the heteroduplexes, phages with deletions were obtained in addition to wild-type phages (Miyada et al., 1982; Adelman et al., 1983; Chan & Smith, 1984). To generate a set of larger deletions for sequence analysis an oligonucleotide was annealed to the lacZ' polylinker region thus restoring a double-stranded DNA with restriction enzyme cleavage sites. Exonucleolytic digestion of the single-stranded 3' end and sealing after another oligonucleotide hybridization yielded an array of deletions (Dale et al., 1985). Mutagenesis of defined DNA segments cloned into filamentous bacteriophage DNA has been achieved by bisulphite treatment of the isolated DNA (Weiher & Schaller, 1982; Warburton et al., 1983), by reaction with methoxylamine (Kadonaga & Knowles, 1985), by misincorporation of α-thiodeoxynucleotides with DNA polymerase I (Abarzúa & Marians, 1984) or by misincorporation of deoxynucleotides with a reverse transcriptase (Mott et al., 1984).

Base changes can then be determined by sequencing or, more conveniently, point mutations can be screened by solution hybridization with a labelled oligonucleotide probe and subsequent gel electrophoresis (Hobden et al., 1985). Single base substitutions in a DNA fragment located near a GC-rich sequence can be analysed directly by gel electrophoresis (Myers et al., 1985). The fragment is then usually transferred into its previous environment. To circumvent the tedious sequence determination to screen possible mutants the lac α-complementation has been used for detection of base changes. Inserts causing frameshifts in the lacZ' reading frame may carry base deletions or insertions after mutagenesis thereby normalizing the reading frame and producing blue plaques (Traboni et al., 1982). To introduce specific base changes at defined sites a more complex arrangement was used (Traboni et al., 1983). The phage DNA (master template) to be mutagenized contains an insert causing a lacZ' frameshift (white plaques). A purified priming segment with the 3' end adjacent to the site of interest normalizes the frameshift of the master template and allows forced misincorporation of the terminal nucleotide by extension of the 3' end with reverse transcriptase, which lacks 3' exonuclease activity, the proof-reading function of bacterial DNA polymerase. Then normal DNA synthesis in the presence of all four nucleotides is allowed. After transformation blue plaques show a high frequency (up to 85%) of specifically mutated sequences.

**Nucleic acid hybridization**

An advantage of cloning DNA into the genome of filamentous bacteriophage is natural strand selection. The strand of a DNA segment linked to the viral strand will be packaged into phage particles. To facilitate oriented cloning in both directions pairs of M13 vectors for α-complementation have been developed, which contain unique restriction sites in an antiparallel order (Messing & Vicira, 1982; Kieny et al., 1983; Norrander et al., 1983). Cloning of DNA in
both directions will therefore result in strand separation being achieved *in vitro*, although on re-infection of bacteria with phages, the double-stranded replicative form can be used to screen for information on each strand (Looney *et al.*, 1984). The orientation of inserts in M13 derivatives was applied for instance in order to determine the coding strand for transcription of the T-DNA expressed in plant tumour cells (Willmitzer *et al.*, 1983) and to analyse transcripts in animal cells (Eggerding & Pierce, 1983; Winberry *et al.*, 1983; West *et al.*, 1984) and transcripts from yeast cells (Breter *et al.*, 1983). Inserts without vector sequences were recovered as single-stranded DNA from phages after the insert had been cloned in self-annealing sequences with suitable restriction sites (Patton & Chae, 1982; Been & Champoux, 1983). A similar stem–loop DNA was primed with oligonucleotides complementary to the insert in order to synthesize highly labelled DNA which was derived exclusively from the inserted DNA, and not from vector sequences (Ricca *et al.*, 1982). Highly labelled DNA complementary to the insert has been separated by gel electrophoresis and used for S1 nuclease mapping of mRNA (Burke, 1984). Hybridization of single-stranded genomic DNA from an M13 vector to cellular RNA was used for mapping exon–intron junctions in the drosophila alcohol dehydrogenase gene (Henikoff, 1983). After S1 digestion the RNA of the hybrid fragment was hydrolysed and the remaining DNA hybridized to single-stranded genomic DNA of the opposite strand. These hybrids were then analysed for their boundaries.

Other applications have been proposed for DNA labelling in hybridization studies. The primer used hybridizes beyond the 5' end of the insertion site of the foreign DNA. DNA synthesis by DNA polymerase invades the M13 part of the DNA leaving the insert single-stranded. Without denaturation the probe will hybridize to DNA complementary to the insert (Hu & Messing, 1982), and cross-linking with psoralen will stabilize the attachment of the labelled DNA fragment to the template (Brown *et al.*, 1982). If a sequencing primer (hybridizing before the 3' end of the insert) is used, the insert will become labelled and may be separated from the template before hybridization to RNA or DNA (Antoniou *et al.*, 1985). The segment of DNA will be synthesized to a defined length if a second primer with a chain-terminating nucleotide is annealed beyond the 5' end of the insert (Liu *et al.*, 1986).

**Conclusions**

Modified filamentous bacteriophages are mainly used for DNA cloning as a source of single-stranded DNA. Their replication origin has also been inserted into pBR plasmids, thus creating vectors which replicate in the ColE1 mode, but can be packaged upon infection with a helper phage (Table 2e). Besides the increased stability of long inserts many improvements introduced for pBR vectors have been made accessible for conversion of these vectors into the single-stranded form. New surveys of methodological advances in cloning and sequencing with plasmid/phage M13/fI chimeric vectors (Vieira & Messing, 1986) and a related phage, Ike (Konings *et al.*, 1986) will be published this year. A different type of vector derived from filamentous bacteriophage has been designed by using the phage fD origin of replication in conjunction with resistance genes (Geider *et al.*, 1985). These pfd plasmids require the expression of viral gene 2 protein in the carrier cells. They can substitute for pBR vectors which can be unsuitable for transformation of eukaryotic cells by expressing a 'poison sequence' (Lusky & Botchan, 1981) or which can interfere with genomic DNA in hybridization experiments. Some of the pfd vectors are not homologous to pBR DNA and most of them can be removed efficiently from carrier cells by temperature shift.

Filamentous bacteriophage can be purified easily and is therefore a convenient source of small or large amounts of single-stranded DNA. *In vitro* synthesis of DNA from filamentous bacteriophage is usually undertaken at the stage of conversion from single-stranded DNA to double-stranded DNA, which is then transformed into competent cells. *In vitro* replication of dsDNA can lead to several rounds of single strand synthesis (Meyer & Geider, 1982) and might be applied for asymmetric segregation of mutated DNA, as shown for chimeric f/phiX DNA replicated *in vitro* in the phage phiX174 mode (Abarzúa & Marians, 1984). Net synthesis of viral double-stranded DNA *in vitro* was achieved with phage phiX174 DNA (Arai *et al.*, 1981). For small inserts chimeric vectors with the phiX174 and ColE1 replication origins (van der Ende *et al.*, 1986)
1982) might be promising for enzymic DNA propagation and subsequent packaging in vitro (Aoyama et al., 1983). Net synthesis of nucleic acids has also been achieved at the RNA level by using Qβ RNA as vector. Inserts could be propagated by Qβ replicase, exceeding by far the input RNA (Miele et al., 1983), but one should be aware of the increasing copy errors during continuous replication of the whole RNA population.

A further extension in the use of single-stranded DNA from filamentous bacteriophages may come from the applications of class IIS restriction endonucleases (Podhajska & Szybalski, 1985), which require a double-stranded recognition sequence and cleave adjacent DNA at a precise distance of several nucleotides. An oligonucleotide with a base-paired recognition sequence and a single-stranded region for annealing with a known sequence in the vector insert would allow cleavage at all anticipated sites. This may be useful for introducing defined changes anywhere in a cloned sequence.

Filamentous bacteriophage has the unique capacity to adjust its particle size to the length of DNA replicated in the viral mode. That makes it a versatile vector for cloning and packing of DNA. Other filamentous viruses having hosts different from E. coli such as phage Pf3 of Pseudomonas aeruginosa (Luiten et al., 1985) may also be modified to cloning vectors provided their hosts can be efficiently transformed or if their genome can be transferred as part of a shuttle plasmid from E. coli to the other bacterium.

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