Cloning of Pf3, a Filamentous Bacteriophage of *Pseudomonas aeruginosa*, into the pBD214 Vector of *Bacillus subtilis*

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Received 3 January 1983/Accepted 12 April 1983

The genome of Pf3, a filamentous single-stranded DNA bacteriophage of *Pseudomonas aeruginosa* (a gram-negative organism) was cloned into pBD214, a plasmid cloning vector of *Bacillus subtilis* (a gram-positive organism). Cloning in the gram-positive organism was done to avoid anticipated lethal effects. The entire Pf3 genome was inserted in each orientation at a unique BclI site within a thymidylate synthetase gene (from *B. subtilis* phage ß22) on the plasmid. Additional clones were made by inserting EcoRI fragments of Pf3 DNA into a unique EcoRI site within this gene.

The phage Pf3, which has *P. aeruginosa* PAO1 (RP1) as host (9), is one of a large number of filamentous phages which infect different gram-negative bacteria. Pf3, like fd and its close relatives f1 and M13, as well as Pf1, lfl, lKe, and Xf, contains a single-stranded circular DNA packed in a slender protein sheath and it infects its host without causing cell lysis (see reviews in reference 2). Various comparative studies have shown that different filamentous viruses have different structures even though their overall morphologies and functions are similar. To pursue comparative studies it is desirable to have suitable cloning systems. Cloning of phage DNAs in vectors of their host bacteria or related bacteria can lead to lethal effects from certain highly expressed phage-encoded proteins. One strategy which obviates this type of problem is to clone into vectors of organisms in which the genes of interest are not expressed, such as in the cloning of DNA of gram-negative organisms into vectors of gram-positive organisms (3, 6). A new positive selection system in gram-positive *B. subtilis* (4) was chosen for cloning the Pf3 genome. The plasmid vector pBD214, approximately 5.7 kilobases (kb), contains a chloramphenicol resistance (*Cm*) gene and a phage ß22 thymidylate synthetase (*thy*) gene. BD393 is a competent thyA thyB host. The vector contains single sites for several restriction enzymes and at least four are in the thy gene, thus allowing convenient ligation of foreign DNAs. Inactivation of this thy gene by any inserted DNA renders the cell resistant to trimethoprim (*Tmp*), and therefore clones of BD393 containing recombinant plasmids can grow on media containing chloramphenicol and trimethoprim. We report here our experience with this system as it may be of value to others.

Pf3 viral DNA is replicated in the host cell via a closed-circular double-stranded intermediate form, Pf3 RF DNA, which we purified by a protocol that included gel electrophoresis in low-melting-point agarose. By screening for restriction enzymes which cleave circular duplex Pf3 DNA relatively few times, and then by carrying out single, double, and some triple digests, we obtained the restriction map shown in Fig. 1. Two of the enzymes, BclI and EcoRI, cleave pBD214 in the thymidylate synthetase gene and were used for cloning.

Cloning of the entire Pf3 DNA was done by ligating a mixture of 2 µg of pBD214 DNA and 6 µg of Pf3 DNA in 40 µl of buffer, each DNA having been linearized at its single BclI site, and then by using dilutions of the ligated mixture to transform BD393. No colonies appeared in controls without added DNA or with Pf3 RF DNA, but the control with the vector DNA alone showed a usual background level of 45 colonies from 10⁷ cells plated. When the ligated DNA mixture was used, 8 × 10⁵ colonies were obtained from 10⁷ cells. The yield in terms of DNA was about 2 × 10⁶ recombinants per µg of plasmid DNA.

Twelve clones were chosen at random for characterization, and gel electrophoresis of their crude lysates showed, in addition to host chromosomal DNA, the presence of supercoiled and relaxed circular plasmid DNA molecules. The recombinant plasmids in six of the clones appeared to be about 12 kb in size, whereas in the other six they appeared to be about 6 kb. Mechanisms giving rise to the latter recombinants are not understood. The larger recombinant plasmids, however, appeared to contain the entire genome of Pf3 oriented in either way. This was shown with EcoRV, a restriction enzyme which,
Cloning by means of the three EcoRI sites on Pf3 DNA (Fig. 1) was done similarly. The ligation of a mixture of the EcoRI-linearized vector and the three Pf3 fragments (4.1, 1.16, and 0.71 kb) was carried out at a 1:1 weight ratio of Pf3 DNA to plasmid DNA and at a total concentration of 100 μg/ml. The efficiency of transformation by the ligation mixture was about $5 \times 10^7$ recombinants per μg of plasmid DNA. Of 50 clones picked and tested, 49 were of the Cm$^r$ Trp$^+$ Lys$^+$ Thy$^+$ Tmp$^+$ phenotype, whereas one was Cm$^s$ Trp$^+$ Lys$^+$ Thy$^+$ Tmp$^+$ phenotype. The occurrence of the latter phenotype is not understood. Agarose gel electrophoresis analysis of the clones with the desired phenotype showed a plasmid larger than the original vector in each of 12 clones tested, and these were of four different size classes (Fig. 3). Further analysis of 12 clones (10 of those in Fig. 3 and 2 others) showed one recombinant plasmid containing both the 4.1-kb and the 1.16-kb Pf3 DNA fragments, one with both the 1.16-kb and the 0.71-kb fragments, four with the 4.1-kb fragment only, and six with the 1.16-kb fragment only (data not shown). A clone containing the largest Pf3 EcoRI fragment was examined in some detail. Its DNA has been used to program an in vitro protein synthesizing system to test for the presence of structural genes and has been also used for DNA sequencing (D. G. Putterman, work in progress).

Expression of gram-negative genes is generally blocked in gram-positive organisms, presumably because of improper interaction of mRNA with BglI, cleaves both Pf3 and pBD214 at a single site. The EcoRV site on pBD214 is 0.1 kb from its BglI site, and in Pf3 the EcoRV site is 1.0 kb from its BglI site. Thus, if the entire genome of Pf3 (5.96 kb) were cloned into pBD214 (~5.7 kb) by means of the BglI sites, the circular recombinant plasmid would be ~11.7 kb in size and would contain two EcoRV sites. Cleavage of it by EcoRV would produce either two fragments of 5.0 and 6.7 kb or two fragments of 1.1 and 10.6 kb, depending on the orientation of the Pf3 DNA. This appears to have occurred, as shown by the data of Fig. 2. From the asymmetry of the EcoRV fragments and the sums of their sizes we concluded that the entire genome of Pf3 was inserted into pBD214, in both orientations.

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with the ribosomes, but transcription may also be altered (6). This essential feature allows, in principle, cloning of any genes coding for proteins lethal to gram-negative organisms in hybrid plasmids. This has been done for some genes of T7 in B. subtilis (8) and now for the PFI genome in the pBD214-BD393 positive selection system. Lethality problems are of concern in PFI cloning even though wild-type PFI infection of P. aeruginosa PAO1 (RP1), like wild-type fd infection of male strains of Escherichia coli, does not lead to lysis or death of the host cells. However, infection by fd phage mutated in any but one or two genes caused the host cells to die (7). It was therefore thought that cloning PFI into a vector such as pBR322 might well lead to lethal effects and loss of intact genes for some of the structural proteins, and we chose at the outset to bypass this possible difficulty by using a gram-positive cloning system. After our work was well underway, we learned that attempts to clone the coat protein and the single-stranded DNA binding protein genes of fd (fd) into pBR322 led to lethal effects, but that stable clones in a plasmid from a gram-positive organism could be obtained (J. Boeke and F. Barany, personal communication). The stable cloning of the entire genome of PFI, either intact or in three EcoRI fragments, demonstrates the utility of the pBD214-BD393 system for use in studies of the PFI genome and those of other filamentous phages as well.

One possibility for future studies presented by the hybrid system is the use of filamentous phage genes to package one strand or the other of pBD214. Insertion of PFI (or fd) packaging signal sequences as well as replication control regions into pBD214 might provide stable cloning, packaging, and strand-separation shuttle vectors. Such hybrid plasmids would be constructed to be stable in B. subtilis and P. aeruginosa and also to package either of the two strands, depending on the orientation of the plasmid DNA. Also, this system may be useful for the construction of PFI phages of various lengths for use in physical studies, as it is known that the length of a filamentous phage virion is determined by the size of the DNA packaged (5).

We thank Paula D. Boyle for preparation of phage stocks and Louise Dennis for help in preparing the manuscript. This work was supported by grants from the National Institute of Allergy and Infectious Diseases.

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