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

DEBRA GLUCK PUTTERMAN,* THOMAS J. GRYCZAN, DAVID DUBNAU, AND LOREN A. DAY

The Public Health Research Institute of the City of New York, New York, New York 10016

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 *Bcl*I site within a thymidylate synthetase gene (from *B. subtilis* phage β22) on the plasmid. Additional clones were made by inserting *Eco*RI fragments of Pf3 DNA into a unique *Eco*RI 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, If1, IKe, 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^r) gene and a phage β22 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^r), 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, *Bcl*I and *Eco*RI, 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 *Bcl*I 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 10^7 cells plated. When the ligated DNA mixture was used, 8×10^3 colonies were obtained from 10^7 cells. The yield in terms of DNA was about 2×10^5 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 *Eco*RV, a restriction enzyme which,
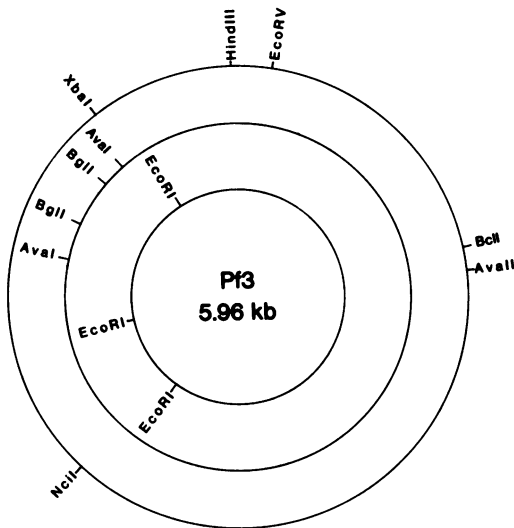
FIG. 1. Restriction map of Pf3 bacteriophage DNA. Outer, middle, and inner circles show sites for enzymes cutting the circular DNA at one, two, and three sites, respectively. Molecular weight calibrations with fragments of ϕX174, PM2, and λ DNAs gave a genome size which was in agreement with the value of 5.96 kb obtained by analytical ultracentrifugation (1).

like BclI, cleaves both Pf3 and pBD214 at a single site. The EcoRV site on pBD214 is 0.1 kb from its BclI site, and in Pf3 the EcoRV site is 1.0 kb from its BclI site. Thus, if the entire genome of Pf3 (5.96 kb) were cloned into pBD214 (~5.7 kb) by means of the BclI 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.

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 5×10^5 recombinants per μg of plasmid DNA. Of 50 clones picked and tested, 49 were of the Cm^r Trp$^-$ Lys$^-$ Thy$^-$ Tmp^r phenotype, whereas one was Cm^r Trp$^-$ Lys$^-$ Thy$^+$ Tmp^r. 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
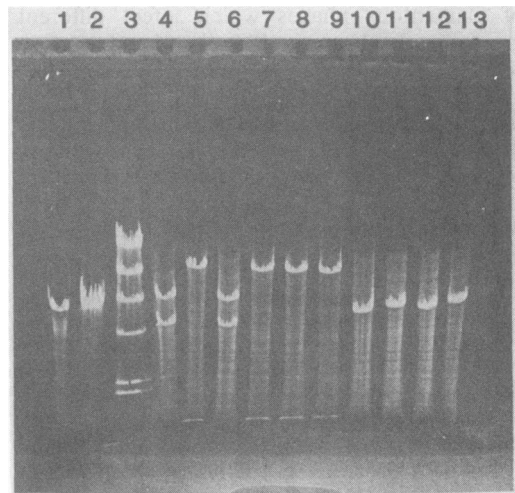

FIG. 2. Size and orientation of Pf3 DNA in recombinant plasmids prepared by using BclI. The DNAs were digested with EcoRV and run on a 0.8% agarose gel. Lanes 1 and 2 are, respectively, Pf3 RF DNA and pBD214 DNA cut with EcoRV. Lane 3 is a set of size standards (23.1, 9.4, 6.56, 4.37, 2.32, and 2.03 kb) from a HindIII digestion of λ DNA. Size estimates in this range from this gel are uncertain to $\pm$0.3 kb; fragments <2 kb appear systematically large. Lanes 4 through 13 are EcoRV digests of Pf3-pBD214 recombinant plasmids. The apparent sizes of the two fragments in lanes 4 and 6 average 6.8 and 5.0 kb, and the apparent sizes of the two fragments in lanes 5, 7, 8, and 9 average 10.2 and ~1.6 kb, according to distance of electrophoresis from the origin. In each case, the sum of the two fragments approximates the size expected for a recombinant plasmid containing the entire Pf3 genome (see text). Lanes 10 through 13 show EcoRV digests of recombinants from which most of the Pf3 genome, including an EcoRV site, had been deleted.
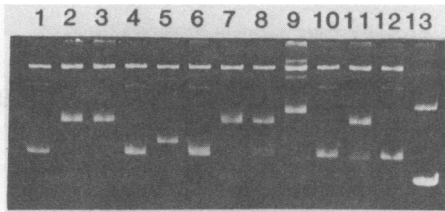
FIG. 3. Analysis of recombinant plasmids in crude lysates from 12 clones obtained from transformation of BD393 with ligation products of a mixture of pBD214 DNA and Pf3 DNA, each digested with EcoRI, on a 0.8% agarose gel. Lanes 1, 4, 6, 11, and 12 show the presence of plasmids of one size class; lanes 2, 3, and 7, a second size class; lane 5, a third; and lane 9, a fourth. Lanes 8 and 11 each show the presence of plasmids of two sizes; such clones were not studied further. Lane 13 is pBD214 DNA alone. The intense band in lanes 1 through 12 at low mobility is chromosomal DNA.

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 here for the Pf3 genome in the pBD214-BD393 positive selection system. Lethality problems are of concern in Pf3 cloning even though wild-type Pf3 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 Pf3 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 f1 (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 Pf3, either intact or in three EcoRI fragments, demonstrates the utility of the pBD214-BD393

system for use in studies of the Pf3 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 Pf3 (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 Pf3 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).

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