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Versatile Cloning Vector for *Pseudomonas aeruginosa*

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A pBR322:RSF1010 composite plasmid, constructed in vitro, was used as a cloning vector in *Pseudomonas aeruginosa*. This nonamplifiable plasmid, pMW79, has a molecular weight of 8.4×10^6 and exists as a multicopy plasmid in both *P. aeruginosa* and *Escherichia coli*. In *P. aeruginosa* strain PAO2003, pMW79 conferred resistance to carbenicillin and tetracycline. Characterization of pMW79 with restriction enzymes revealed that four enzymes (*Bam*HI, *Sal*I, *Hin*dIII, and *Hpa*I) cleaved the plasmid at unique restriction sites. Cloning *P. aeruginosa* chromosomal deoxyribonucleic acid fragments into the *Bam*HI or *Sal*I site of pMW79 inactivated the tetracycline resistance gene. Thus, cells carrying recombinant plasmids could be identified by their carbenicillin resistance, tetracycline sensitivity phenotype. Deoxyribonucleic acid fragments of approximately 0.5 to 7.0 megadaltons were inserted into pMW79, and the recombinant plasmids were stably maintained in a recombination-deficient (*recA*) *P. aeruginosa* host.

Genetic analysis of *Pseudomonas aeruginosa* has been hampered by a lack of hybrids of plasmids and the bacterial chromosome. Although some success has been achieved using the IncP1 group R-factors as carriers of chromosomal genes (9, 10, 11), the size of such R-prime plasmids (>40 megadaltons) makes their in vitro manipulation difficult.

Molecular cloning offers an attractive alternative for isolating chromosomal gene sequences. We have used the well-characterized vector pBR322 (3, 17) to clone *P. aeruginosa* genes into *Escherichia coli* (D. O. Wood, F. L. Macrina, and P. V. Phibbs, Jr., Proceedings of the Second Annual Mid-Atlantic Regional Extrachromosomal Elements Meeting, Plasmid 2: 301, 1979). However, some genes of *P. aeruginosa* may not be expressed in an *E. coli* background (12), and, unfortunately, vectors comparable to pBR322 have not been available in *Pseudomonas*. spp. Recently, Bagdasarian et al. (2) described the construction of several hybrid plasmids, derived from plasmid RSF1010 (8), which can be used as cloning vectors in *Pseudomonas* spp. They also described the construction of a potentially more versatile vector composed of plasmids pBR322 and RSF1010 but were unable to introduce it into *Pseudomonas* spp. In this paper, we describe the construction of a similar pBR322:RSF1010 plasmid hybrid, which can be maintained in a recombination-deficient (*recA*) strain of *P. aeruginosa*. This plasmid was found suitable for use as a cloning vector in this species.

The bacterial host strains and plasmids used in this study are listed in Table 1. Plasmids were isolated and purified by a modification of the method of Clewell and Helinski (6). DNA samples were subjected to electrophoresis in a standard vertical slab gel apparatus by the method of Meyers et al. (15). Restriction endonuclease digestions and ligation were performed as directed by the supplier (Bethesda Research Laboratories, Rockville, Md.). Transformation of *E. coli* was accomplished by the method of Brown et al. (4), whereas the procedure used to transform *P. aeruginosa* was a combination of two published protocols (4, 14). Plasmid copy numbers were determined by subjecting labeled ([*methyl*-^{3}H]thymidine for *E. coli* and [5-^{3}H]cytidine for *P. aeruginosa*) total cell lysates to cesium chloride-ethidium bromide equilibrium centrifugation.

A recombinant plasmid was constructed by mixing *Eco*RI-digested pBR322 and RSF1010, ligating the plasmids, and transforming them into *E. coli* C600. Transformants exhibiting resistance to both ampicillin and streptomycin were selected and screened for plasmid DNA. Transformants containing only a single plasmid species were identified. One of these transformants, MOB79, containing the plasmid designated pMW79, was selected for further study.

Digestion of pMW79 with *Eco*RI yielded the two expected fragments (Fig. 1A) that migrated in a manner indistinguishable from those of *Eco*RI digests of pBR322 and RSF1010 (data not shown). Thus, the molecular weight of

TABLE 1. *Bacterial strains and plasmids used*[a]

Stock no.	Genotype or phenotype	Source
A. Bacterial strain		
E. coli		
C600	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ^- F^-	P. Shipley
C600(RSF1010)	C600 carrying RSF1010	P. Shipley
WS110	*leu pro thi hsdM hsdR lacY* Str^r carrying pBR322	H. Boyer via B. Steinberg
MOB79	C600 carrying pMW79	This study
P. aeruginosa		
PAO2003	*argH32 str-39 cml-2 rec-2* FP^-	V. Krishnapillai (5)
AC161	*leu-38 res-10* $FP2^+$	A. Chakrabarty
V388	Mucoid	F. Macrina (13)
MOB82	PAO2003 carrying pMW79	This study
MOB93	PAO2003 carrying a recombinant plasmid; DNA inserted into *Sal*I site of pMW79	This study
MOB94	PAO2003 carrying a recombinant plasmid; DNA inserted into *Bam*HI site of pMW79	This study
MOB95	PAO2003 carrying a recombinant plasmid; DNA inserted into *Bam*HI site of pMW79	This study
B. Plasmid		
pBR322	Ap, Tc	Bolivar et al. (3)
RSF1010	Sm/Su	Guerry et al. (8)
pMW79	Ap/Cb Tc Sm	This study

[a] Genotype symbols are described by Bachmann and Low (1). Ap, Tc, Sm, Su, and Cb refer to resistance to ampicillin, tetracycline, streptomycin, sulfonamide, and carbenicillin, respectively. *res* refers to a restrictionless phenotype of *P. aeruginosa*.

pMW79 was calculated to be 8.4×10^6, based on a molecular weight of 2.9×10^6 for pBR322 (17) and 5.5×10^6 for RSF1010 (16). This figure is in good agreement with the molecular weight of pMW79 ($8.6 \pm 0.5 \times 10^6$) determined by comparing migration distance of the plasmid to that of known standards during agarose gel electrophoresis.

To determine whether some of the more commonly used unique restriction enzyme sites of pBR322 and RSF1010 were present in the chimera, pMW79 was also digested with *Bam*HI, *Hin*dIII, *Sal*I, *Hpa*I, and *Pst*I (Fig. 1). With the exception of *Pst*I (Fig. 1B), all of the tested enzymes cleaved pMW79 at unique sites, generating a single band of pMW79 unit length (Fig. 1D to G). *Pst*I cleaved pMW79 into four fragments (Fig. 1B). The arrow in Fig. 1 marks the position of two small, faint *Pst*I fragments.

pMW79 isolated from *E. coli* C600 was introduced into *P. aeruginosa* PAO2003 via transformation at a frequency of approximately 10^{-6}. pMW79 isolated from *P. aeruginosa* (MOB82) transformed PAO2003 at a 10-fold-higher frequency. When present in PAO2003, pMW79 conferred resistance to carbenicillin (500 μg/ml) and tetracycline (25 μg/ml). Selection for one of these resistance determinants was required to prevent loss of pMW79 from the *Pseudomonas* host.

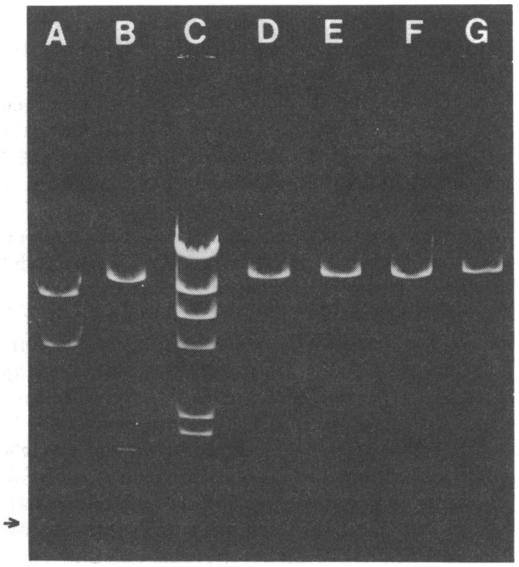

FIG. 1. *Agarose gel electrophoresis analysis of pMW79 digested with: (A) EcoRI; (B) PstI; (D) HindII; (E) BamHI; (F) SalI; (G) HpaI. λ digested with HindIII (C) was included as linear molecular weight markers; top to bottom these are 15×10^6, 6.4×10^6, 4.3×10^6, 2.9×10^6, 1.6×10^6, and 1.4×10^6. The agarose concentration was 0.8%, and migration was from top to bottom. The arrow indicates the position of two small, faint PstI fragments.*

pMW79 existed in multiple copies in both *E. coli* and *P. aeruginosa* (15 and 30 copies per genome equivalent, respectively). Although Gautier and Bonewald (7) have recently described a pBR322:RSF1010 hybrid that can be amplified in *E. coli*, pMW79 was not amplifiable. Based on restriction maps, the orientation of the two plasmids in the hybrids differed. This suggests that the orientation of RSF1010 and pBR322 in pMW79 results in the use of the RSF1010 replicator and not the pBR322 replicator which is responsible for amplification (3).

The vector properties of pMW79 were examined by cleaving the vector with either *Bam*HI or *Sal*I and mixing it with similarly digested *P. aeruginosa* chromosomal DNA (extracted from strain V388). Both enzymes cleaved pMW79 within the tetracycline gene. The DNAs were ligated and mixed with competent cells of a *recA* strain of *P. aeruginosa* (5), and portions were spread on L-plates containing carbenicillin (500 μg/ml). The resulting carbenicillin-resistant transformants were then tested for resistance to tetracycline. Approximately 6% of the carbenicillin-resistant transformants were found to be sensitive to tetracycline and, when examined for plasmid DNA, contained plasmids larger than pMW79. In Fig. 2, three clones (MOB93, MOB94, MOB95), two generated by *Bam*HI cleavage (Fig. 2C, 2E) and one by *Sal*I cleavage (Fig. 2G), are presented. Digestion of these clones by the appropriate enzyme generate two fragments (Fig. 2D, 2F, 2H), one of which corresponded to linear pMW79. The arrow shows the position of the small fragment in lane H. The amount of DNA inserted ranged from approximately 0.5 to 7.0 megadaltons.

Although pMW79 is usable as a cloning vector in *P. aeruginosa*, efforts are under way to reduce its size. Restriction endonuclease mapping has revealed that the *Pst*I sites of RSF1010 map near one another (2; this study). A reduction in the size of pMW79 by the elimination of a major portion of the RSF1010 molecule would not only make the vector more convenient to use, but also could result in the elimination of these sites. The remaining *Pst*I site located on the pBR322 portion of the plasmid would then be available for cloning. This would be a significant improvement of pMW79 since the carbenicillin resistance gene would then be available for insertional inactivation.

Our results suggest that maintenance of a pBR322:RSF1010 hybrid in *P. aeruginosa* requires a RecA$^-$ host. Bagdasarian et al. (2) were unable to transform a restrictionless (*res*) but RecA$^+$ strain of *P. aeruginosa* with their analogous hybrid. We also were unable to introduce

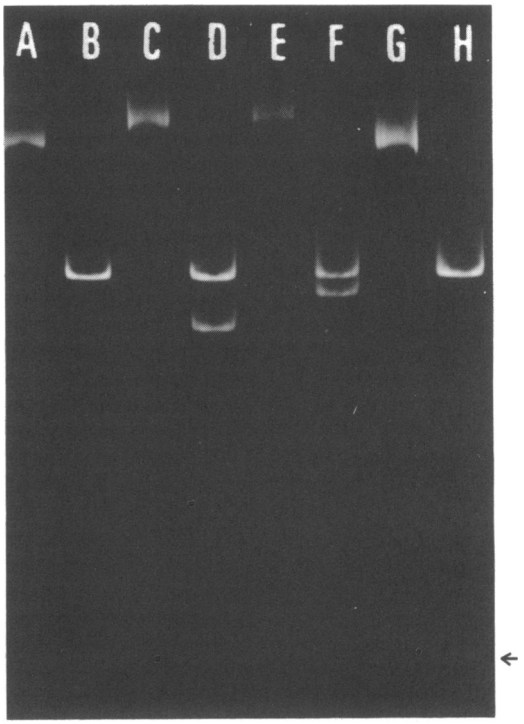

FIG. 2. *Agarose gel electrophoresis analysis of (A) undigested pMW79; (B) pMW79 digested with BamHI; (C) plasmid DNA from MOB94; (D) plasmid DNA from MOB94 digested with BamHI; (E) plasmid DNA from MOB95; (F) plasmid DNA from MOB95 digested with BamHI; (G) plasmid DNA from MOB93; (H) plasmid DNA from MOB93 digested with SalI. The agarose concentration was 0.8%, and migration was from top to bottom. The arrow shows the position of a small fragment in lane H.*

pMW79 into this strain, possibly because of RecA$^+$-induced excision of pBR322. Since the cloning of homologous *P. aeruginosa* DNA into a *P. aeruginosa* host would require a RecA$^-$ recipient to prevent loss of the cloned fragment through recombination, the inability of pMW79 to be maintained in RecA$^+$ hosts does not preclude its use as a cloning vector. Low-molecular-weight variants of pMW79 would provide cloning vectors for *Pseudomonas* spp. of comparable versatility to those of *E. coli*.

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