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Development of Broad-Host-Range Vectors and Gene Banks: Self-Cloning of the *Pseudomonas aeruginosa* PAO Chromosome

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A host-vector system for *Pseudomonas aeruginosa* PAO was developed. Scattered regions of the strain PAO chromosome were cloned by direct selection for complementation of auxotrophs or from a DNA gene bank which contains over 1,000 independently isolated chromosome-vector recombinant plasmids. The use of partially digested chromosomal DNA facilitated the selection of a variety of strain PAO chromosomal markers. The progenitor of the vector was a small, multicopy plasmid, pRO1600, found in a PAO strain which had acquired RP1 in a mating experiment. The bacterial host range that could be determined by transformation of vectors produced from pRO1600 resembles that for plasmid RP1. Two derivative plasmids were formed: pRO1613, for cloning DNA cleaved with restriction endonuclease *Pst*I, and pRO1614, which was formed by deleting part of pRO1613 and fusion with plasmid pBR322. Plasmid pRO1614 utilizes known cloning sites within the tetracycline resistance region of pBR322.

Molecular cloning of chromosomal DNA with plasmids developed for that purpose has been reported for a variety of bacterial species. In most instances, the utility of the plasmid cloning vector derives from the inactivation of an antibiotic resistance determinant of the vector as a consequence of the insertion of a piece of hetero-DNA from the chromosome into a site on the vector cleaved by a restriction endonuclease. Thus, the continuity of the vector gene, and hence its expression, is interrupted by the insertion of foreign pieces of DNA. One of the most widely used plasmid vectors of this kind is plasmid pBR322, which has the advantage of a single restriction endonuclease site within its antibiotic resistance determinants for ampicillin and tetracycline (2). However, the host range of pBR322 is limited to *Escherichia coli* and related bacterial strains, which prohibits its usefulness in bacteria with disparate properties. Bagdasarian et al. (1) and recently Wood et al. (23) have reported the construction of a cloning vector for *Pseudomonas aeruginosa* to overcome this difficulty. Another plasmid vector also has been developed by Ditta et al., which was derived from the broad-host-range plasmid RK2 (8). This system offers the advantage of potential for use in a wide variety of gram-negative bacterial species in view of its broad host range.

We report here the derivation of another cloning vector system from a particular *P. aeruginosa* strain which contains plasmid RP1. Plasmid RP1 is similar, if not identical, to plasmid RK2 which was used previously for this purpose and also to plasmid RP4 (3). One of our vectors, designated pRO1613, allows cloning of DNA that was previously digested with *Pst*I by direct selection for the desired phenotype. Another vector, designated pRO1614, was derived from a deleted pRO1613 and the insertion of pBR322. Using these plasmids, we show the cloning of widely scattered regions of the *P. aeruginosa* PAO chromosome and suggest a protocol for the construction of a gene bank of the PAO chromosome. This gene bank was used for the selection of a wide variety of recombinant plasmids which complement mutations in the chromosome of strain PAO.

MATERIALS AND METHODS

Bacterial strains and plasmids. The relevent properties of the bacterial strains and the plasmids used in this study are listed in Table 1.

Media. Minimal medium (VBG) and complex medium (TN) were prepared as described previously (19). When nutritional selection for transformants was done, amino acid requirements were satisfied by the addition of these components to a final concentration of 0.5 mM. Antibiotic supplements were as described in the footnotes to the tables. Selection or indirect testing for the acquisition of catabolic markers was done using a minimal medium (MMO) described previously for this purpose (22). Carbon sources were incorporated into MMO at a final concentration of 0.2%.

Preparation of DNA. Plasmid DNA was prepared

TABLE 1. Bacteria and plasmids

Bacterial strain/plasmid	Genotype[a]	Reference or source
Bacterium		
PAO1	Prototroph	21
PAO2	*ser-3*	21
PAO25	*argF leu-10*	21
PAO236	*hisII ilvB,C lys-12 met-28 proA trpC,D,E*	11
PAO2003	*argH recA*	5
PAO2178	*catA1 met-9020 nar-9011*	21, —[b]
PAO2198	*arg-90310 catA1 chu-9002 leu-9014 lys-9015 met-9020 nar-9011 trpA,B*	—[b]
PAO2324	*catA1 met-9020 nar-9011 puuD6 tyu-9009*	—[b]
PAO2369	*catA1 cnu-9001 met-9020 nar-9011 puuE8 tyu-9025*	—[b]
GMA052	*hisV*	—[c]
GMA057	*hisIV*	—[c]
GMA065	*hisIII*	—[c]
GMA253	*cys-5605 hisI*	21, —[c]
P. putida PPO131	*his-1*	—[d]
P. fluorescens PFO141	*his-1*	18, —[e]
E. coli ED8654	*met hsdR hsdM*	—[f]
K. pneumoniae KPM100	Prototroph	—[g]
Plasmid		
RP1	Cb^r Tc^r $PRR1^s$ $PRD1^s$	19, 20
pBR322	Cb^r Tc^r	2
pRO1600	Cryptic	This study
pRO1601	pRO1600::Tn*1* Cb^r	This study
pRO1613	Cb^r	This study
pRO1614	Cb^r Tc^r	This study

[a] Marker abbreviations. Bacterial strains: *arg*, arginine; *cat*, catechol utilization; *chu*, choline utilization; *cnu*, carnosine utilization; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine-valine; *leu*, leucine; *lys*, lysine; *met*, methionine; *nar*, nitrate reductase; *pro*, proline; *puu*, purine utilization; *rec*, recombination; *hsdR hsdM*, *E. coli* K-12 restriction and modification; *ser*, serine; *trp*, tryptophan; *tyu*, tyrosine utilization. Plasmids: Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; $PRR1^s$, sensitive to phage PRR1; $PRD1^s$, sensitive to phage PRD1.

[b] Matsumoto collection (14), received from P. Phibbs, Medical College of Virginia, Richmond.

[c] Lee collection (15), received from J. Loper, University of Cincinnati Medical School, Cincinnati, Ohio.

[d] Mutant of ATCC 12633.

[e] Mutant of strain PFO14 isolated from milk and received from J. Jezeski, University of Minnesota, Minneapolis.

[f] Strain K-12, received from D. Jackson.

[g] Stock culture, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor.

using a modification of the method of Guerry et al. (10). Cells were grown overnight on the surface of TN agar medium plates. In some cases, carbenicillin (0.5 mg/ml) was included in the medium to maintain selection for plasmids. The cells were harvested from the surface of the plates by adding 5 ml of sterile water to each plate and scraping with a glass rod. These suspensions were decanted and pooled in a bottle which was shaken vigorously to disperse clumps of cells. The cell suspension was then centrifuged at ambient temperature, and the pellets were suspended in TS buffer (9% sucrose–0.05 M Tris, pH 8; 10 ml of buffer for each centrifuge tube with about 1.5×10^{10} cells). The following additions and manipulations were then done with each suspension. Disodium EDTA (0.5 M, pH 8.0) was added to a final concentration of 0.08 M. This was followed immediately by the addition of lysozyme (10 mg/ml in 0.25 M Tris, pH 8.0) to a final concentration of 0.77 mg/ml. The tubes were then briefly mixed on a Vortex mixer and incubated at 37°C for 5 min. After this, sodium dodecyl sulfate (15% in distilled water) was added to a final concentration of 2%. The tubes were inverted slowly several times to mix and then incubated in a water bath for 5 min at 37°C. During this time they were removed several times and slowly inverted to promote lysis of the cells. Finally, 5 M NaCl in distilled water was added to a final concentration of 0.95 M and mixed into the suspension by several gentle inversions. The tubes were then placed into an ice water bath for 10 min followed by storage overnight at 4°C. Precipitation of chromosome, harvest of plasmid DNA, and CsCl-ethidium bromide centrifugation were then done as described previously by Hansen and Olsen (12). DNA was stored frozen in TO buffer (10 mM Tris–1 mM disodium EDTA, pH 8) and thawed slowly in ice water when used.

Recombinant plasmids were surveyed for their size with cells harvested from a patch of growth on selective medium and lysis of cells by the procedure of Hansen and Olsen (12).

Chromosomal DNA was harvested from *P. aeruginosa* PAO1 which had been selected for resistance to

rifampin (50 μg/ml) in our laboratory. The procedure described above (modification of Guerry et al.) was used for chromosomal DNA also except that the lysed cell suspensions were given two 30-s pulses at full speed on a Vortex mixer before the addition of 5 M NaCl. This was done to fragment the chromosome. These lysed suspensions were then salt precipitated followed by DNA precipitation and CsCl-ethidium bromide centrifugation as described previously for plasmid DNA (12).

Transformation. Pseudomonads were transformed using a modification of the method of Mercer and Loutit (16). Bacteria were grown overnight on TN agar, and a portion was then inoculated into TN broth with incubation for 2 to 3 h, reaching a cell density of 10^8 per ml. The cells were centrifuged at 4°C, and the pellet was suspended in 0.5 volume of cold, sterile $MgCl_2$ (0.15 M in distilled water). The pellet was dispersed and held in an ice water bath for an additional 5 min. The cells were centrifuged, and the pellet was suspended as before but then held in the ice water bath for 20 min. The cells were centrifuged again, and the pellet was suspended in 0.1 volume of cold $MgCl_2$ (0.15 M). Transforming DNA (10 to 50 μl) was placed in a cold centrifuge tube, and 0.2 ml of the above cells was added with mixing. This mixture was incubated in an ice water bath for 60 min followed by a heat pulse in a water bath at 37°C for 3 min while the tube was gently swirled. The DNA-cell mixture was then placed immediately in an ice water bath and incubated for 5 min. After this, 0.5 ml of TN broth was added and the suspension was incubated at 37°C for 1 to 2.5 h. The cells were then plated on selective medium, and the plates were incubated at appropriate temperatures for 48 h.

E. coli and *Klebsiella pneumoniae* were transformed by using a modification of the method of Davis et al. (7). For this, bacteria were grown as above and cultures were chilled in an ice water bath for 10 min at the end of the growth period. The cells were then centrifuged at 4°C, and the pellets were suspended in 0.5 volume of CTG buffer (50 mM $CaCl_2$, 10% glycerol, 50 mg of thymidine per ml). The cells were dispersed and incubated for 5 min in a bath at 0°C. They were then centrifuged, and the pellets were suspended in 0.05 volume of the original culture. Cells (0.2 ml) were added to tubes which contained 20 to 100 μl of DNA and mixed; these were held for 3 min in an ice water bath. The tubes were then transferred to a 45°C water bath and slowly swirled for 2 min; they were then placed in an ice water bath for 5 min. After this, 0.5 ml of TN broth was added and the mixture was incubated at 37°C for 0.5 to 2.5 h. Samples of these transformation mixtures were then plated at 37°C on selective medium. For both transformation procedures, all materials, including pipettes, were at 4°C unless otherwise noted.

Enzymes. Restriction endonuclease digestion and ligation were done as suggested by the supplier (Bethesda Research Laboratories, Rockville, Md.). Digested DNA to be ligated was incubated for 20 h at 17°C. Ligations were done in a volume of 20 μl or less. In some experiments, this required concentration of cleaved DNA by ethanol precipitation before the addition of ligase and buffer. These ligation mixtures were used for transformation as described above and in the footnotes to the tables.

RESULTS

Development of a cloning vector system. We have routinely transferred plasmid RP1 from one bacterium to another, and on one such occasion, after the preparation of plasmid DNA from the recipient, we noted the presence of a plasmid that corresponded to RP1 and another smaller plasmid of approximately 2 Mdaltons in molecular size. The recipient bacterium used for these experiments is plasmid-free, so this result suggests the rare occurrence of an RP1 mutation which occurred during transfer to a recipient. The relationship of these plasmids is shown in Fig. 1. Figure 1, file A, shows RP1 DNA isolated from a typical PAO2(RP1) transconjugant. In file B, a rare PAO2(RP1) transconjugant used for the preparation of plasmid DNA shows two plasmids, RP1 at the top of the gel and a smaller miniplasmid near the bottom which we have designated pRO1600. Its size was 2 Mdaltons in relation to plasmid DNA standards prepared from *E. coli* V517 (13) shown in file D. We used the DNA preparation shown in file B to transform PAO25 with selection for the acquisition of either carbenicillin (Cb^r), tetracycline (Tc^r), or kanamycin (Km^r) resistance. The rationale here

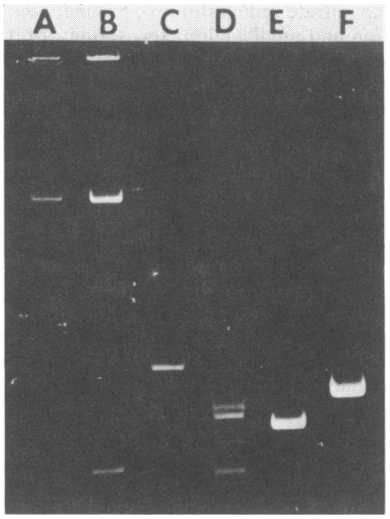

FIG. 1. Agarose gel electrophoresis showing plasmids RP1, pRO1600, pRO1601, pRO1613, and pRO1614. Their origin and derivation are discussed in the text. DNA was prepared as described in the text. A 25-μl sample of each DNA preparation was mixed with 10 μl of dye and subjected to electrophoresis. Samples were as follows: (A) PAO2(RP1)-11; (B) PAO2(RP1/pRO1600); (C) PAO2(pRO1601); (D) DNA from *E. coli* V517, a multiplasmid-containing strain used as a size standard (13); (E) PAO2(pRO1613); (F) PAO2-(pRO1614). Gels which contained 0.7% agarose were constructed and run as described previously (12).

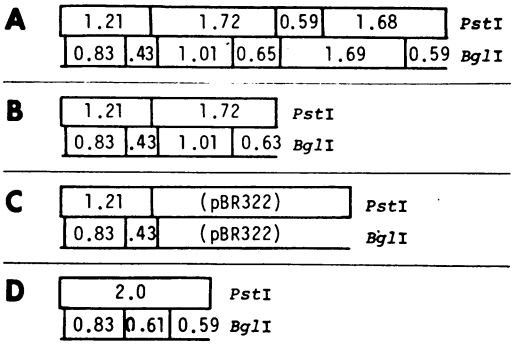

FIG. 2. Physical map of plasmids: (A) pRO1601; (B) pRO1613; (C) pRO1614; and (D) pRO1600. Numerical values represent molecular size in Mdaltons for restriction enzyme cleavage fragments. These were determined using *Hin*dIII cleavage of phage lambda DNA and plasmid pBR322 DNA cleaved with combinations of two enzymes (double digest) which were used in this study.

was that one of these determinants from RP1 might be included within pRO1600 or, alternatively, that a derivative might be obtained of the small plasmid designated pRO1600 which had acquired Tn*1*, a Cb^r transposon, from RP1 in the mixed DNA. When this was done, 8% of the transformants were Cb^r, Tc^s, and Km^s. One such transformant is shown in Fig. 1, file C. It was designated pRO1601, and its size is about 5.2 Mdaltons. This result was consistent with the acquisition of Tn*1* (3.2 Mdaltons) by pRO1600 (2 Mdaltons). Plasmid pRO1601 and other similar Tn*1*-transposed transformants were used to prepare plasmid DNA and were cleaved by *Pst*I or *Bgl*II restriction endonuclease singly or together, and the fragments were subjected to agarose gel electrophoresis. Partial digests were also done to determine contiguity of the fragments. A physical map for pRO1601 is shown in Fig. 2A. Since the digest pattern for Tn*1* was known from previous work (unpublished data), we thus deduced the map of pRO1600 shown in Fig. 2D. Similar determinations on 11 other Cb^r transformants yielded different regions of pRO1600 that were altered as a consequence of a Tn*1* insertion. However, none showed a Tn*1* insertion within the 0.83-Mdalton *Bgl*II fragment. This suggests that this 0.83-Mdalton region may include the replication functions for plasmid pRO1600. Therefore, other regions of pRO1600 may be dispensable, and either the size of pRO1601 may be reduced by *Pst*I digestion, deletion of nonessential *Pst*I fragments, and ligation of the replication region to the fragment with Cb^r, or the replication region could be added to plasmid pBR322, which is nonviable in PAO, to provide replication functions in *Pseudomonas*. Accordingly, pRO1601 was digested with *Pst*I, a portion of this digest was added to pBR322 that was digested with *Pst*I, and the mixture was ligated. These preparations (with and without pBR322) were then used to transform PAO2 (Table 2). Undigested pRO1601 and pBR322 were also used as controls in this experiment. Several transformants from the *Pst*I-digested pRO1601 and the mixture of pRO1601 and pBR322 were purified, and DNA was prepared and analyzed by restriction endonuclease cleavage as above (Fig. 2B and C). In the former case, a plasmid designated pRO1613, which contains only two *Pst*I sites, was obtained, and the *Pst*I site shown at the interior junction of the 1.21- and 1.72-Mdalton fragment occurs within the beta-lactamase determinant. In Fig. 2C, a plasmid designated pRO1614, which contains only the 1.21-Mdalton *Pst*I fragment of pRO1601 and all of pBR322, is shown. In this instance, the beta-lactamase gene of pRO1601, known to have a *Pst*I site within it (2), has apparently matched up with the *Pst*I-cleaved pBR322 beta-lactamase gene to form a viable beta-lactamase determinant. This determinant, therefore, is derived in part from both pRO1601 and pBR322. Other cleavage sites within the region designated pBR322 are as reported previously for pBR322 (2). Thus, pRO1614 can be used for *Bam*HI and *Hin*dIII cloning as reported for pBR322 cloning in *E. coli* (2).

We next determined the bacterial host range of pRO1613 or pRO1614 derived from the ex-

TABLE 2. Derivation of cloning plasmids[a]

Plasmid DNA used	Treatment of DNA	No. of transformants[b]	Antibiotic resistance
pBR322	None	0	
pRO1601	None	69,000	Cb^r
pRO1601	*Pst*I cleavage plus ligation	70	Cb^r
pRO1601 plus pBR322	*Pst*I cleavage plus ligation	2	Cb^r
		4	Cb^r Tc^r

[a] About 0.5 μg of plasmid DNA was treated as shown in column 2 and transformed into PAO2 as described in the text.

[b] Selection was for Cb^r transformants on TN agar medium with 0.5 mg of carbenicillin per ml. Cb^r transformants were picked onto TN agar medium with tetracycline (60 μg/ml).

TABLE 3. Host range of cloning plasmids pRO1613 and pRO1614[a]

Plasmid DNA[b]	Recipient bacteria	Selection[c]	No. of transformants per μg of DNA
pRO1613	*P. aeruginosa* PAO2	Cb^r	132,000
pRO1614	*P. aeruginosa* PAO2	Tc^r	38,000
pRO1614	*P. putida* PPO131	Tc^r	960
pRO1614	*P. fluorescens* PFO141	Tc^r	24,000
pRO1614	*E. coli* ED8654	Tc^r	5,500
pRO1614	*K. pneumoniae* KPM100	Tc^r	1,300

[a] *E. coli* and *K. pneumoniae* were transformed using the CTG buffer procedure described in the text. *Pseudomonas* strains were transformed using the Mercer and Loutit procedure (16).

[b] DNA was prepared from PAO2 which contained either pRO1613 or pRO1614.

[c] Selection was as described in Table 2, footnote *b*, for Cb^r transformants. Tc^r transformants were selected on TN agar medium with tetracycline (60 μg/ml). *P. putida* and *P. fluorescens* were incubated at 25°C. Other strains were incubated at 37°C.

periments shown in Table 2. For this we used several bacterial strains of disparate physiological properties. The choice of either pRO1613 or pRO1614 for the determination was influenced by the intrinsic Cb^r or Tc^r of these strains, which would allow unambiguous scoring of transformants for their acquisition of the antibiotic resistances encoded by the plasmids. The data resulting from this preliminary host range determination are shown in Table 3. The data indicate the broad host range of the plasmids among the pseudomonads, *E. coli*, and *Klebsiella*. Perhaps this list will be extended as transformation procedures are developed for other gram-negative bacterial species that are of interest for molecular cloning. Although the same DNA preparation for pRO1613 or pRO1614 was used in all cases here, the number of transformants obtained varied with the recipient. This may reflect restriction of the plasmid DNA in view of its preparation for strain PAO and perhaps the use of suboptimal transformation procedures for the bacterial strains shown here.

Cloning the PAO chromosome. We tested the utility of pRO1613 and pRO1614 for cloning with *Pst*I-cleaved pRO1613 and the *Pst*I-cleaved PAO chromosome. Similarly, pRO1614 and the chromosome were cleaved with *Hin*dIII or *Bam*HI. The DNA suspensions were mixed after cleavage, ligated, and transformed into PAO236. Strain PAO236 is a multiple auxotroph, and transformants were selected for their nutritional independence. Usually, one or two nutritionally independent transformants were obtained in this way from an experiment, and these were purified, characterized for antibiotic resistance, and used to prepare plasmid DNA for analysis. Plasmid DNA for typical transformants was cleaved with the enzyme used for their preparation and electrophoresed to determine the presence of pRO1613, pRO1614, and chromosomal DNA components. Table 4 shows the size of the cleavage fragments obtained when these typical recombinant plasmids were digested with the same enzyme used for their construction. Plasmids pRO1665 and pRO1661 were formed using the vector pRO1613, and both contain the 1.1-Mdalton region of pRO1613 which contains the replicator and part of the beta-lactamase gene cleaved at its internal *Pst*I site. The nonessential region of pRO1613 (1.8-Mdalton fragment) was lost as expected. However, fragments unique to

TABLE 4. Composition of PAO recombinant plasmids

Recombinant plasmid designation	PAO236 marker	Enzyme used	Fragment sizes (Mdaltons)[a]
pRO1613	Vector	*Pst*I	1.8, 1.1
pRO1665	*lys-12*	*Pst*I	6.1, 1.1, 0.74
pRO1661	*met-28*	*Pst*I	2.4, 1.1
pRO1614	Vector	*Bam*HI	3.9
pRO1655	*proA*	*Bam*HI	3.9, 2.3
pRO1657	*proA*	*Bam*HI	12.1, 3.9, 2.3
pRO1658	*hisII*	*Bam*HI	13.6, 3.9, 0.82
pRO1615	*ilvB,C*	*Hin*dIII	3.9, 1.8, 1.1, 0.95, 0.74, 0.50

[a] Fragment sizes were calculated as described in the text for data in Fig. 2.

the recombinant plasmids, pRO1665 and pRO1661, were obtained. These fragments presumably represent DNA which complements either the *lys-12* or *met-28* mutation. In the case of pRO1665, two unique fragments were obtained although it is uncertain whether these are derived from a contiguous region of the chromosome or whether they represent the random association of two different fragments during the cloning process.

When pRO1614 was used as the vector plasmid, cleavage of chromosomal and plasmid DNA with *Hin*dIII or *Bam*HI resulted in the formation of the recombinant plasmids pRO1655, pRO1657, pRO1658, and pRO1615, which showed unique fragments when analyzed with the same enzyme used for their formation. For all of these recombinant plasmids, a 3.9-Mdalton fragment representing the vector was obtained plus the inserted chromosomal DNA. Plasmid pRO1655 shows a single fragment (2.3 Mdalton) which complements the *proA* mutation of PAO236. This 2.3-Mdalton fragment also was obtained for pRO1657, another *proA* recombinant plasmid. However, in this case another chromosomal fragment was also cloned. More than one unique band derived from recombinant plasmids pRO1658 and pRO1615 also was observed. To further confirm the authenticity of these recombinant plasmids, plasmid DNA was extracted from the bacteria used for their selection and was used to transform PAO236 with selection for the relevant marker. As expected, a high frequency of transformation, corresponding to that for either pRO1613 or pRO1614 plasmid DNA, by Cb^r selection, occurred. The sum of the fragment sizes for pRO1658 is about 18.3 Mdaltons, representing 14.4 Mdaltons of the PAO chromosome. Recombinant plasmids of this size are near the upper limit of those obtained to date with direct selection for the acquisition of nutritional markers. Not all of the recombinant plasmids described here are stable in the absence of nutritional and antibiotic selection in appropriate mutant bacteria. For example, pRO1615 requires both selection for *ilvB,C*$^+$ and Cb^r for maintenance in PAO236. If Cb^r selection is omitted, a nutritionally independent chromosomal recombinant occurs; if *ilvB,C* selection is omitted, a deleted version of pRO1615 occurs (data not shown). However, the other plasmids described in Table 4, and for the most part all others obtained to date, are stable in *recA*$^+$ PAO bacteria in the absence of nutritional or antibiotic selection on TN agar maintenance medium.

Derivation of a chromosomal gene bank for PAO. Over a period of 2 years, the number of recombinant plasmids obtained using direct selection and the enzymes described in the foregoing work was small in comparison with the total yield of transformants obtained using antibiotic selection. When the transformants that were selected for antibiotic resistance were scored for insertional inactivation of an antibiotic resistance marker resulting from the addition of chromosomal DNA, few were found. Therefore, the cloning efficiency was low. Two reasons for this may be relevant: the donor chromosomal DNA may be poorly cleaved by the enzymes used, the use of *recA*$^+$-transforming recipient bacteria may diminish the recovery of recombinant plasmids, or both. We investigated the former possibility by digesting PAO chromosomal DNA with the enzymes used in the foregoing work (Fig. 3). These digests show variable cleavage of the fragmented chromosomal DNA preparation (described above).

On the basis of the result shown in Fig. 3, file E, we prepared a *Bam*HI digest of fragmented DNA from PAO to be used in evaluating a possible enhancement in cloning efficiency with the use of PAO2003, a mutant of PAO which is *recA* (5). For this we also cleaved pRO1614 with *Bam*HI and selected PAO2003 transformants for their acquisition of Cb^r. These Cb^r transformants were then streaked onto medium which contained tetracycline to determine insertional inactivation of the Tc^r region of the vector as a consequence of the inclusion of a chromosomal fragment at its *Bam*HI cleavage site. When this

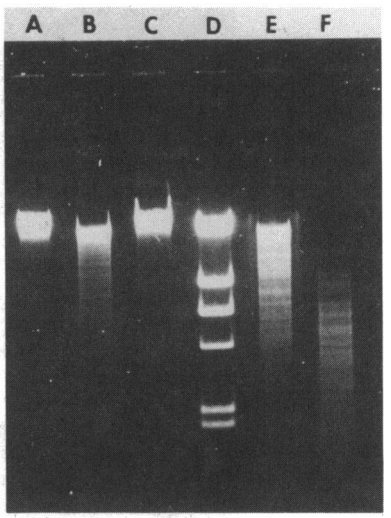

FIG. 3. Agarose gel electrophoresis of cleaved PAO chromosomal DNA. Samples were cleaved as described in the text and run as described in the legend to Fig. 1. Samples were as follows: (A) uncleaved DNA; (B) chromosome cleaved with *Hin*dIII; (C) chromosome cleaved with *Sal*I; (D) lambda cleaved with *Hin*dIII; (E) chromosome cleaved with *Bam*HI; (F) chromosome cleaved with *Pst*I.

was done, 132 of 552 colonies picked were Tc^s (24%). In another experiment, DNA from PAO was prepared from a rifampin-resistant mutant (50 μg/ml), and 958 of 3,090 transformant colonies picked were Tc^s (31%). Therefore, these data support the utility of the *recA* strain (PAO2003) for cloning in *P. aeruginosa* as suggested previously by Carbon et al. for cloning in *E. coli* (4). In the second set of experiments described above, we also did direct selection for the acquisition of rifampin resistance or an $argH^+$ recombinant plasmid complementing the *argH* mutation in the chromosome of PAO2003. Two recombinant plasmids of different size were found for rifampin resistance; one $argH^+$ recombinant plasmid was observed. As in all previous examples of recombinant plasmids discussed here, DNA from PAO2003 transformants containing these plasmids was used to retransform PAO2003, and the relevant markers were acquired at a high frequency that was independent of selection for either Cb^r or the selective marker. Therefore, these were authentic recombinant plasmids for the markers relevant to their initial selection.

The 132 Tc^s transformants from the first experiment described above were patched onto three TN agar plates (44 per plate) which contained carbenicillin (0.5 mg/ml). The 958 Tc^s clones obtained in the second experiment described above were patched among 10 TN agar plates. After overnight incubation, the cells were washed from each agar plate, and 0.5 ml of each plate wash was used to inoculate 10 nutrient agar plates which contained carbenicillin. The 13 groups of 10 TN agar plates (per inoculum source) were harvested and extracted for plasmid DNA which was subsequently centrifuged in CsCl-ethidium bromide gradients. The plasmid DNA band was harvested from each of the 13 gradients and electrophoresed to determine their heterogeneous content of recombinant plasmids (Fig. 4). Figure 4, files A, B, and C, show plasmid DNA preparations derived from a mixed culture resulting from the inoculum which contained 44 independently isolated

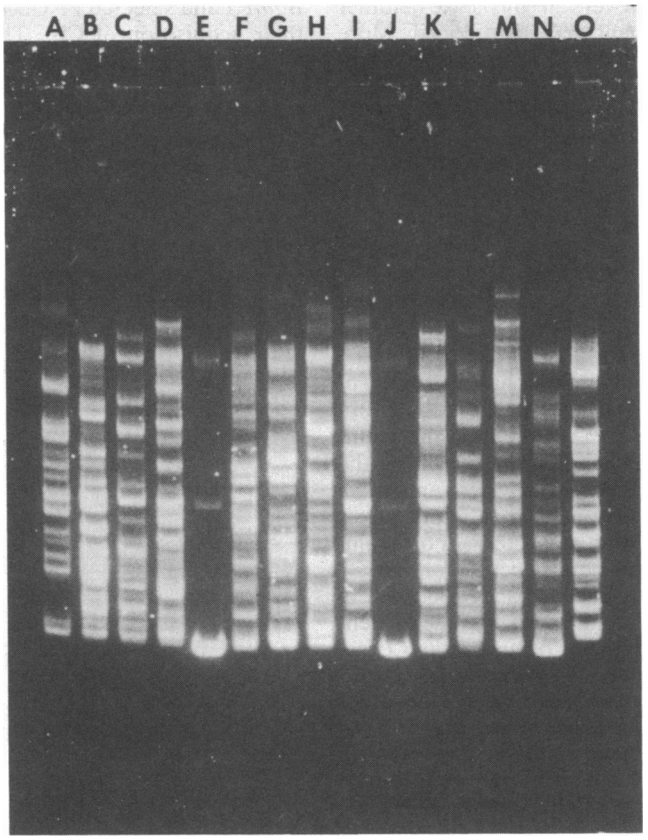

FIG. 4. Agarose gel electrophoresis of recombinant plasmid gene banks derived from the PAO chromosome. DNA for the pooled recombinant plasmids was prepared as described in the text. Samples in files E and J are the vector, pRO1614. Other files show plasmid DNA obtained from cultures inoculated from mixed suspensions which contained independently isolated recombinant plasmids as described in the text.

pRO1614 Tc^s transformants. The other mixed-culture DNA preparations were derived from inocula prepared from 100 Tc^s transformant colonies. None of the files show unique plasmids corresponding to the number of transformants used for their preparation. However, throughout the files, some plasmid bands are more prominent than others, perhaps indicative of either higher-copy-number recombinant plasmids at those locations or alternatively, several different recombinant plasmids of approximately the same size. We favor the second alternative in view of the utility of these plasmid DNA gene banks for selecting almost any marker tested to date when various mutants are used as transforming recipient bacteria. Also, when these DNA gene banks are used as a source of transforming DNA with direct selection for a given marker, the frequency of transformation varies widely for various markers. This suggests that some recombinant plasmids may be present in low concentration and not visualized on the agarose gel shown in Fig. 4.

A summary of the recombinant plasmid phenotypes isolated from the earlier work with direct selection and also from the gene banks shown in Fig. 4 is presented in Table 5. In Table 5, only one recombinant plasmid for each phenotype is listed. However, in most instances, several recombinant plasmids for a given phenotype were isolated, and they vary in size for their independent isolations; the larger versions show more than one *Bam*HI cleavage site when plasmid DNA is isolated and digested from the various isolates of the same phenotype. As shown earlier in Table 4, however, a band unique to the marker in question is found in all recombinant plasmids of the same phenotype. In some cases, selection for one marker results in the isolation of recombinant plasmids which also include adjacent markers. For example, plasmids pRO1680, pRO1669, and pRO1690, which were selected for *nar-9011*, *met-9011*, and *trpA,B*, respectively, were subsequently tested for adjacent markers shown on the Royle et al. map (21) and were found to complement these too. On the other hand, recombinant plasmids have been selected which encode for these markers singly. These results suggest that the conditions used for the digestion of chromosomal DNA result in incomplete digestion of the chromosome, allowing for the isolation of fragments of variable length from the same region of the chromosome.

TABLE 5. Representative recombinant plasmids of the PAO chromosome[a]

PAO strain and plasmid designation	Markers complemented
PAO25(pRO1703)	*argF*
PAO236(pRO1615)[b]	*ilvB,C*
PAO236(pRO1616)[b]	*met-28*
PAO236(pRO1654)[b]	*lys-12*
PAO236(pRO1657)[b]	*proA*
PAO236(pRO1658)[b]	*hisII*
PAO2003(pRO1700)[b]	*rifA*
PAO2003(pRO1702)[b]	*argH*
PAO2178(pRO1669)[b]	*catA met-9011*
PAO2198(pRO1687)	*chu-9002*
PAO2198(pRO1689)	*lys-9015*
PAO2198(pRO1690)	*chu-9002 trpA,B*
PAO2198(pRO1694)	*leu-10*
PAO2324(pRO1677)	*puuD*
PAO2324(pRO1683)	*met-9011*
PAO2369(pRO1679)	*nar-9011*
PAO2369(pRO1680)	*nar-9011 tyu-9009*
PAO2369(pRO1682)	*catA*
GMA052(pRO1707)	*hisIV*
GMA057(pRO1705)	*hisV*
GMA065(pRO1713)	*hisIII*
GMA253(pRO1704)	*hisI*

[a] The derivation of the mixed-plasmid DNA suspensions is discussed in the text.

[b] These recombinant plasmids were obtained using appropriate PAO nutritional mutants. They were isolated using direct selection from cleaved chromosome and vector DNA that was ligated.

The recombinant plasmids listed in Table 5 correspond to scattered regions of the PAO chromosome. This is evident from Fig. 5, where their map location corresponding to the chromosomal mutations that they complement is shown. For this we used the most recent map of Royle et al. as a guide to position the markers in relation to a time-of-entry map, using PAO sex factors and transductional analysis linkage determinations (21). Most of the plasmids listed in Table 5 and Fig. 5 were obtained from the gene pools shown in Fig. 4. These results suggest that procedures for the preparation of the chromosome, cleavage with *Bam*HI, and indirect selection for the isolation of recombinant plasmids, followed by the preparation of recombinant DNA from mixed cultures, are suitable as a general protocol. These procedures apparently do not bias against the molecular cloning of significantly extensive regions of the PAO chromosome.

DISCUSSION

There is precedent for large plasmids producing mutant derivative plasmids of small size and altered incompatibility and copy number relationships toward their progenitor plasmids. For example, Goebel and Bonewald have reported the occurrence of miniplasmids from R1*drd*-19B2 in wild-type *E. coli* K-12 (9). These miniplasmids were found to have a high copy number in relation to their progenitor, R1*drd*-19B2, and

the miniplasmids were comaintained with R1*drd*-19B2. Therefore, the miniplasmids were not only deletion mutants of the parental plasmid, but they were also mutant in their incompatibility properties. In the light of more recent knowledge (17), these miniplasmids probably arose as a consequence of their segregation from the parental plasmid during DNA replication, with cleavage of the parental plasmid occurring between the origin of replication and replication control functions. Alternatively, cleavage at the origin of replication may have produced an altered recognition site for the regulation of DNA synthesis by regulatory metabolites encoded by the parental plasmid. In any case, the traits reported for these miniplasmids correspond to those for pRO1600, and therefore this suggests that a similar, if not identical, mechanism may have produced pRO1600. Further details concerning the composition and functional mapping of pRO1600 are the subject of another report (R. H. Olsen, G. DeBusscher, and W. R. McCombic, manuscript in preparation).

In a previous report, Clarke and Carbon sheared the *E. coli* chromosome and then formed hybrid plasmids in vitro with poly(deoxyadenylate-deoxythymidylate) "connectors" (6). This was done to diminish the likelihood of recovering chromosomal fragments which contained cleavage sites in structural genes and to obviate the need for a cloning site in the vector which would be insertionally inactivated with the addition of a cloned fragment of chromosome. In this report, we attempted to minimize the recovery of DNA fragments cleaved in structural genes by devising a procedure which reproducibly results in partially digested chromosomal DNA. The degree to which the *P. aeruginosa* chromosome is cleaved by various restriction endonucleases may be related to the high (68%) guanine-plus-cytosine content in the organisms. The utility of this is suggested by our isolation of recombinant plasmids with additional *Bam*HI sits within the *Bam*HI-cloned DNA. Therefore, if a particular allele contained a cleavage site within its structure, partial digests enhanced the recovery of an intact allele. In this regard, the map distribution (Fig. 5) of recombinant plasmids listed in Table 5 suggests that we isolated partially cleaved fragments of chromosomal DNA from disparate regions of the PAO chromosome. Furthermore, the use of *Bam*HI for this purpose may have promoted the recovery of diverse recombinant plasmids in view of the extent of cleavage of the chromosome by this enzyme and the distribution of *Bam*HI cleavage sites on the chromosome.

In summary, the bacterial host range of

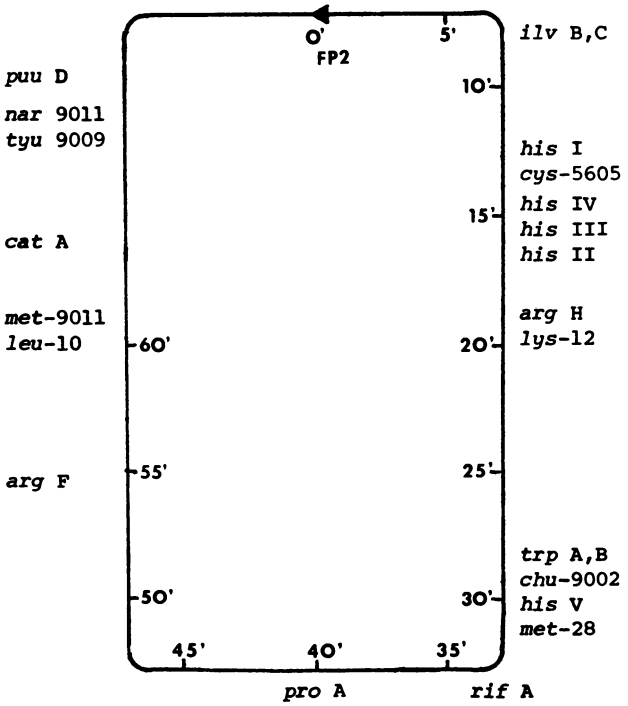

FIG. 5. Chromosomal map of PAO showing the map location of markers complemented by representative recombinant plasmids listed in Table 5. The map locations are those of Royle et al. (21). Marker abbreviations are listed in Table 1, footnote *a*.

pRO1613 and pRO1614 includes *E. coli*, *P. aeruginosa*, and other pseudomonads and thus suggests the possible utility of these cloning vectors for use in gram-negative bacterial strains that are unrelated regarding their ecological niche or salient physiological properties. However, to facilitate this, it may be necessay to introduce other antibiotic resistance markers to allow selection in bacterial genera that are intrinsically resistant to carbenicillin. Furthermore, the utility of pRO1613 is limited to the cloning of DNA fragments which can be recovered by direct selection for an acquired trait. Further studies are in progress to obviate these limitations.

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