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Transposable Plasmid Deoxyribonucleic Acid Sequence in *Pseudomonas aeruginosa* Which Mediates Resistance to Gentamicin and Four Other Antimicrobial Agents

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A 9.1 × 10^6-dalton transposable deoxyribonucleic acid sequence resides within *Pseudomonas aeruginosa* plasmid R1033 and mediates resistance to gentamicin, streptomycin, sulfamethoxazole, chloramphenicol, and mercuric chloride. Transposability was demonstrated in *Escherichia coli* when this sequence, designated Tn*1696*, excised from R1033 and integrated into plasmid pMB8. Excision and insertion of Tn*1696* occurred independently of the host Rec phenotype and may involve the 140-base pair, inverted deoxyribonucleic acid repeated region that flanks this sequence. Occurrence of a multiresistance transposon on a transferrable plasmid that has a broad host range may have serious epidemiological and therapeutic consequences.

Plasmids of *Pseudomonas aeruginosa* are classified according to their ability to coexist within the same host cell. Those which cannot reside together usually share a high degree of DNA sequence similarity and are considered to be within the same incompatibility group (12). Of the eleven known plasmid incompatibility groups observed for *Pseudomonas*, plasmids of the P-1 class, which was the first reported, commonly mediate resistance to ampicillin (Ap), tetracycline (Tc), and kanamycin (Km) (but not gentamicin [Gm]) and have a broad host range (11). Recently Smith et al. (19) isolated a P-1-type plasmid (R1033) that mediates gentamicin resistance by specifying production of an enzyme which modifies the drug by acetylation. This type of gentamicin-modifying enzyme is commonly observed in plasmid-bearing clinical isolates of *P. aeruginosa* (15), many of which harbor plasmids of other incompatibility groups (11, 14, 17, 21). It has been suggested that acquisition of gentamicin resistance by a plasmid of this incompatibility group occurs through a recombination event involving a sequence of DNA that transposes the gentamicin resistance gene from another plasmid (11, 19, 21). This report describes the molecular nature of a transposon, designated Tn*1696*, that resides within R1033 and mediates resistance to gentamicin and four other antimicrobial agents.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* J53-2(R1033) was the source of purified R1033 plasmid DNA (19) and was kindly provided by R. W. Hedges. *E. coli* C600 and JC2926 *recA* were used as recipients in transformation (1). A streptomycin-resistant (*strA*), nalidixic acid-resistant (*nalA*) variant of P3748 (*polA*; 5), provided by J. Karam, was employed as a recipient for conjugation experiments. C600(pMB8) was kindly provided by R. Gill.

R1033 is a 45 × 10^6-dalton (45 Md) plasmid of the P-1 incompatibility group of *P. aeruginosa* and mediates resistance to ampicillin, kanamycin, tetracycline, gentamicin, streptomycin (Sm), sulfamethoxazole (Su), chloramphenicol (Cm), and mercuric chloride (Hg) (19). The ColE1 derivative plasmid pMB8 is 1.72 Md and mediates immunity to colicin E1 protein (2). RSF2001 is a 63-Md derivative of the F plasmid that carries the kanamycin resistance gene (9). RSF1050, used in heteroduplex analysis, is a 5.0-Md colicin E1-immune derivative of pMB8, which contains Tn3 and mediates ampicillin resistance (9). Plasmid pCER100 was derived and isolated during this study and is described below.

Transformation and plasmid isolation. Transformation of $CaCl_2$-treated *E. coli* was carried out as described previously (3). Dye-buoyant density cesium chloride-ethidium bromide centrifugation was utilized for purifying covalently closed circular plasmid DNA from crude lysates (7).

Determination of colicin E1 and antimicrobial susceptibility. Sensitivity to colicin E1 was performed by transferring single colonies with toothpicks or streaking individual colonies to Trypticase soy agar (BBL Microbiology Systems) plates containing 0.5 ml of colicin E1 extract per 100 ml of Trypticase soy agar (6). Susceptibility to gentamicin, chloramphenicol, streptomycin, sulfamethoxazole, and mercuric chloride was determined as described previously (8).

Assay for chloramphenicol acetyltransferase. Cell extracts were prepared and assayed by the spectrophotometric technique of Shaw (18).

Conjugation experiments. Mating between strain JC2926 (harboring plasmids RSF2001 and pCER100) and recipient P3478 (*nalA polA*) was performed for 2 h by the procedure of So et al. (20). Transcipients were selected on MacConkey agar (Difco Laboratories) containing 5 μg of gentamicin per ml and 50 μg of nalidixic acid per ml.

Heteroduplex analysis and contour length determination. A 0.1-μg amount of each of the plasmids used for heteroduplex analysis was mixed and treated with 0.5 ng of DNase I (Worthington Biochemicals Corp.) per ml for 5 min (9). Denaturation, renaturation, and spreading of plasmid heteroduplexes were performed by the formamide method of Davis et al. (4). Heteroduplexes were visualized with a Phillips EM 301 electron microscope. Open circular plasmid DNA prepared by DNase I digestion was spread (4), and molecular weights were calculated relative to open circular pMB8 (1.72 Md).

Isolation of pMB8 recombinant plasmids. *E. coli* C600(pMB8) was transformed with purified R1033 plasmid DNA (3), grown for 8 h in fresh Trypticase soy broth (BBL Microbiology Systems), and plated on MacConkey agar containing 5 μg of gentamicin per ml. Gm^r clones were checked for colicin E1 immunity as described above. The plasmid DNA content of four gentamicin-resistant, colicin E1-immune clones was determined by the crude lysate-agarose gel procedure (13). The clone containing both R1033 and pMB8 was used for isolation of pMB8 recombinant plasmids.

C600(R1033, pMB8) was grown to the late log phase in 1 liter of brain heart infusion broth (BBL Microbiology Systems). At this point, 250 μg of chloramphenicol per ml was added to inhibit protein synthesis and promote relaxed replication of pMB8 (2) and any recombinants that may have formed between the two coexisting plasmids. Multiple copies of pMB8 and pMB8-R1033 composite plasmids can be obtained relative to R1033, since this large plasmid requires protein synthesis for replication. Therefore, this technique served as an amplification of the proportion of PMB8-R1033 recombinants relative to R1033.

After 16 h of amplification, the cells were harvested, and purified covalently closed circular plasmid DNA was isolated from crude lysates (7) by the 10% polyethylene glycol (PEG-6000; Baker) precipitation procedure (10). Two micrograms of the purified plasmid mixture was transformed into C600 with selection for gentamicin resistance. One hundred Gm^r clones were checked for colicin E1 immunity.

RESULTS

Isolation of pMB8 recombinant plasmid pCER100. Purified covalently closed circular plasmid DNA from C600(R1033, pMB8) was utilized for transformation of *E. coli* C600, followed by selection for gentamicin resistance. Due to the larger size of R1033, transformation with this plasmid occurred at a lower frequency than with pMB8 recombinant plasmids in the mixture; so, selection of the latter plasmids was favored. The presence of the recombinant plasmid in the Gm^r transformants was confirmed by demonstrating their immunity to colicin E1.

Eight gentamicin-resistant, colicin E1-immune transformants were screened for plasmid DNA content, and all clones were found to contain plasmids of approximately 11 Md, including its concatemeric forms. Purified plasmid DNA from one of the clones (Fig. 1A) was subsequently used for transforming JC2926 *recA* to isolate the 11-Md monomeric replicating form (Fig. 1B). This plasmid was designated pCER100.

The fact that the 11-Md plasmid mediated gentamicin resistance and colicin E1 immunity was preliminary evidence that the structural gene for gentamicin resistance resided within a 9-Md DNA sequence which had transposed from R1033 to the smaller pMB8 plasmid.

Antibiotic resistance mediated by pCER100. *E. coli* C600(pCER100) mediated re-

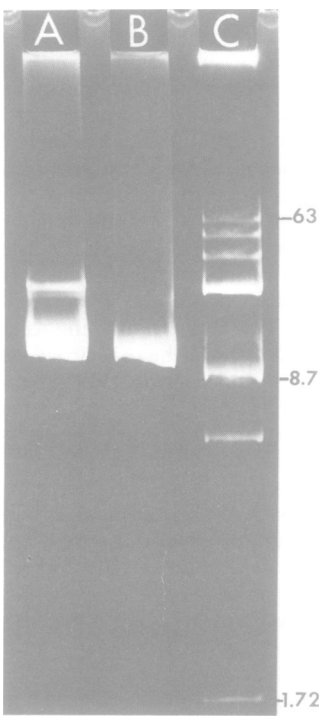

FIG. 1. *Agarose gel electrophoresis of purified plasmid DNA from (A) C600(pCER100), showing concatemeric replicating forms of the 11-Md plasmid, and from (B) JC2926 recA(pCER100), in which 11-Md monomer of pCER100 was isolated; (C) purified plasmid standard DNAs: RSF2001 (63 Md), R1033 (45 Md), RP4 (34 Md), R6K (26 Md), RSF1010:Ap201 (8.7 Md), pSC101 (6.0 Md), and pMB8 (1.72 Md).*

sistance to gentamicin, streptomycin, sulfamethoxazole, chloramphenicol, and mercuric chloride at levels similar to those observed for C600(R1033) (Table 1). All strains were resistant to 40 μg of chloramphenicol per ml, and no acetyltransferase activity was demonstrable in extracts from C600(pCER100) or C600(R1033). This indicated that another chloramphenicol resistance mechanism which did not involve drug modification by acetylation was determined by the 9-Md sequence.

Molecular nature of pCER100. We determined a molecular weight of 10.8 ± 0.48 for pCER100 from measurement of 12 molecules by electron microscopy and concluded that a 9.1(±0.45)-Md DNA sequence had recombined with pMB8. This sequence was designated Tn*1696*.

Heteroduplex analysis showed that pCER100 was indeed a composite formed between R1033 and pMB8. A heteroduplex between pMB8 and pCER100 (pMB8::Tn*1696*) plasmids is shown in Fig. 2. All heteroduplexes demonstrated a double-stranded DNA loop which equaled the size of one pMB8 molecule. The thinner, more twisted single-stranded loop corresponded to the 9.1-Md sequence from R1033. A short double-stranded "stalk" region between the single- and

TABLE 1. *Minimal inhibitory concentrations (MICs) of E. coli* carrying Tn*1696*[a]

E. coli strain	MIC of:				
	Gentamicin (μg/ml)	Streptomycin (μg/ml)	Chloramphenicol (μg/ml)	Sulfamethoxazole (μg/ml)	Mercuric chloride (mg/ml)
C600(R1033)	25	25	40	2,500	20
C600(pCER100)	50	50	40	2,500	40
P3478(RSF2001::Tn*1696*), no. 1	25	12.5	40	1,250	40
P3478(RSF2001::Tn*1696*), no. 2	25	12.5	40	10	40
C600	1.5	3.1	3.1	10	0.5

[a] All determinations were performed by the twofold broth dilution procedure (8).

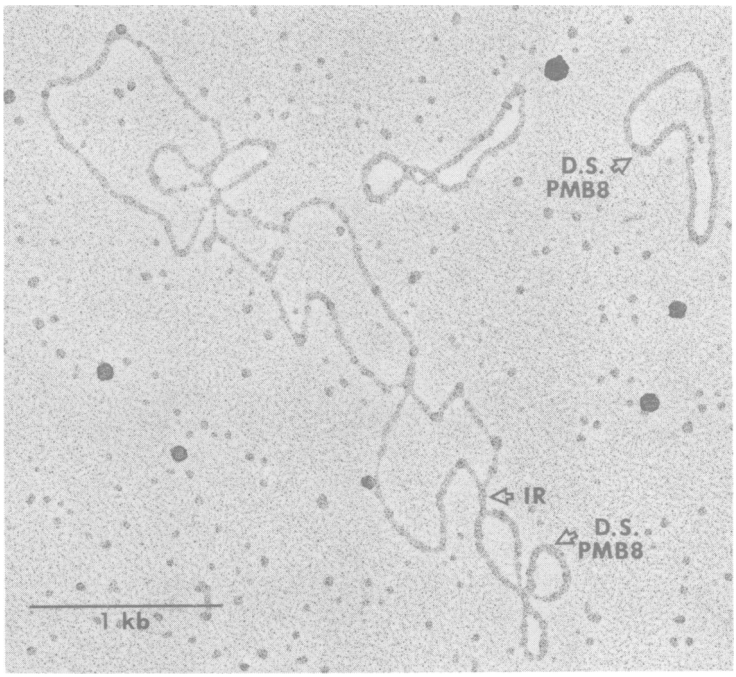

FIG. 2. *Heteroduplex of open circular pMB8 and pCER100. The double-stranded loop (D.S. pMB8) equals in length a single pMB8 molecule (upper right). The large single-stranded loop corresponds to Tn1696 (9.1 Md) which inserted into pMB8. IR, Double-stranded, inverted repeated sequence at the point of insertion in pMB8. kb, Kilobase.*

double-stranded loops was also observed (IR in Fig. 2). These regions have been seen in heteroduplexes of other transposable genetic elements and indicate the presence of inverted repeated regions (16). The inverted repetition in Tn*1696* measured approximately 140 base pairs. The size was confirmed by heteroduplex analysis between RSF1050 (pMB8::Tn*3*) and pCER100. The inverted repetition of Tn*1696* is essentially the same length as that of Tn*3* (approximately 140 base pairs; 16) (Fig. 3).

Transposition independent of host recombination. Recombination by transposable sequences occurs independently of conventional host recombination mechanisms (16). To confirm that Tn*1696* was capable of transposing in the absence of a functional *recA* gene, we utilized a system described by So et al. (20).

pCER100 plasmid DNA was transformed into *E. coli* JC2926 *recA* harboring RSF2001. JC2926(RSF2001, pCER100) was the donor in conjugation experiments with P3478 *polA nalA*, with selection for Gm^r Nal^r transcipients. The pMB8 replication system requires an active *polA* gene and therefore Gm^r Nal^r transcipients would emerge only if Tn*1696* had transposed from pCER100 onto RSF2001 (9, 20).

Crude lysates of eight transcipients were prepared, and portions were layered on an agarose gel. All eight contained a plasmid 9 Md larger than RSF2001, indicating that Tn*1696* had transposed from pCER100 to RSF2001 (Fig. 4). Four of the transcipients were resistant to gentamicin, streptomycin, sulfamethoxazole, chloramphenicol, and mercuric chloride, confirming that RSF2001 had acquired Tn*1696* (Table 1). Four transcipients were Gm^r, Sm^r, Cm^r, and Hg^r but Su^s (Table 1). No difference was observed in

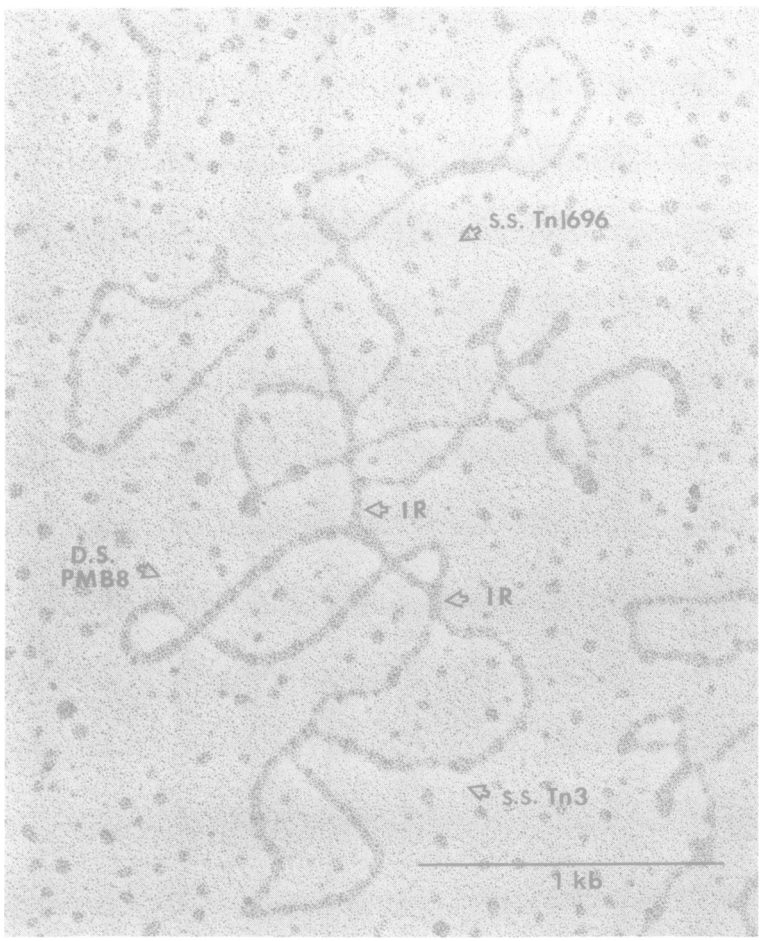

FIG. 3. *Heteroduplex of RSF1050 and pCER100, demonstrating similar-length (140-base pairs) inverted repeats (IR) of Tn1696 and Tn3 inserted in pMB8. S.S., Single stranded; D.S., double stranded; kb, kilobase.*

the size of RSF2001 recombinant plasmids between Su^r or Su^s transcipients as determined by agarose electrophoresis (Fig. 4B and C). The difference in sensitivity to sulfamethoxazole may reflect the location or orientation of insertion of Tn*1696* within RSF2001 (16).

DISCUSSION

Plasmid-mediated resistance to gentamicin, streptomycin, sulfamethoxazole, and mercuric chloride is common in *P. aeruginosa*, especially among the P-2, P-3, and P-6 incompatibility groups observed for this species (17, 21). Investigators (11, 21) have demonstrated the ability of these resistance determinants to recombine en bloc with other plasmids in *Pseudomonas*. Before the discovery of R1033, P-1 plasmids were only observed to mediate resistance to carbenicillin, tetracycline, and kanamycin. Jacoby et al. (11) observed that P-1 plasmids could acquire gentamicin resistance from plasmids of the P-2 incompatibility group and that acquisition of this resistance was usually linked to streptomycin, sulfamethoxazole, and mercuric chloride resistances. The recombination event between P-1 and P-2 plasmids as described by Jacoby et al. (11) is similar to transposition in that it involves integration of a nonhomologous sequence of DNA into the P-1 plasmid, with inactivation of genes in the recipient plasmid. This type of recombination occurs independently of the host Rec phenotype. A similar type of event has also been reported by Stanisich et al. (21), wherein a 14-Md sequence residing on plasmid R26 (P-1 incompatibility group), mediating resistance to gentamicin, streptomycin, sulfamethoxazole, and mercuric chloride, recombined with a nonhomologous recipient plasmid (21).

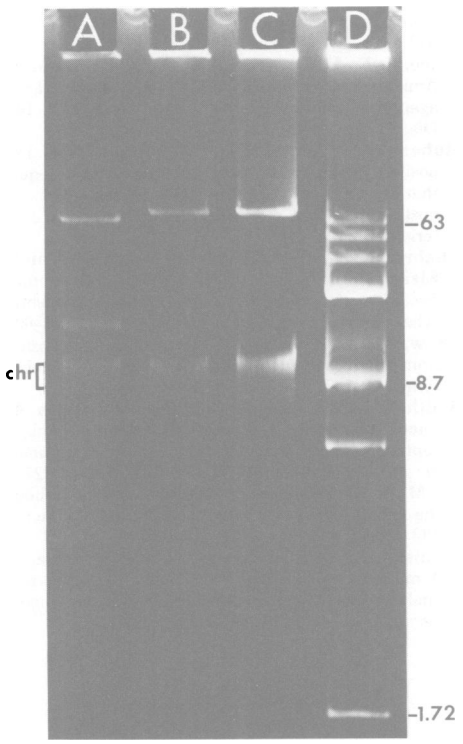

FIG. 4. *Agarose gel electrophoresis of ethanol-precipitated DNA from cleared lysates of (A) JC2926(RSF2001, pCER100), 63 and 11 Md, respectively; (B) P3478(RSF2001::Tn1696) Su^r, 72 Md; (C) P3478(RSF2001::Tn1696) Su^s, 72 Md; and (D) purified, standard plasmid DNAs (see legend to Fig. 1C). Chr, Region where chromosomal fragments have migrated in columns A, B, and C.*

The 9.1-Md transposable DNA sequence described herein mediates resistance to gentamicin, chloramphenicol, streptomycin, sulfamethoxazole, and mercuric chloride and transposes between plasmids independently of the host Rec phenotype. It is logical to assume that R1033 evolved through a transposition event between two *Pseudomonas* plasmids, one of which carried Tn*1696*. Unlike the other sequences observed in recombination experiments between P-1- and P-2-type plasmids, Tn*1696* encodes a chloramphenicol resistance gene in addition to the other four. Chloramphenicol resistance associated with production of chloramphenicol acetyltransferase and not linked with mercuric chloride resistance has been previously reported (11) as being involved in recombinant plasmid formation. In contrast, the chloramphenicol resistance associated with Tn*1696* is linked with mercuric chloride resistance and does not elaborate acetyltransferase, but phenotypically appears similar to the plasmid-mediated impermeability mechanism described (14) for chloramphenicol resistance. Despite the difference in phenotype and molecular size, it would be interesting to determine the relatedness of Tn*1696* and the DNA sequences encoding gentamicin resistance reported by others (11, 21).

The 140-base pair inverted and repeated DNA sequence flanking Tn*1696* is similar to that observed for other transposable antibiotic resistance genes (16). It is unlikely that inverted DNA repetitions spontaneously evolved through random mutation events to bracket five intervening resistance genes. A more plausible theory has been suggested (16), involving the integration of insertion-like sequences (IS*1*, IS*2*, etc.) which flank the resistance determinants and become responsible for their subsequent transposability upon excision.

flank the resistance determinants and become responsible for their subsequent transposability upon excision.

The evolution of Tn*1696* and its emergence on a promiscuous plasmid that can be stably maintained in many gram-negative species has serious epidemiological and therapeutic implications. Heavy selection pressure due to the widespread utilization of gentamicin in the treatment of serious infections could enhance dissemination of Tn*1696* and similar transposable gentamicin resistance genes among plasmid-bearing clinical isolates. This conclusion is supported by the observation of two transposable gentamicin resistance genes in *Serratia marcescens* which integrated into coresident transferrable plasmids and eventually spread to other infecting organisms (C. E. Rubens, W. F. McNeill, and W. E. Farrar, unpublished data).

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LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli*. Bacteriol. Rev. **36**:525-527.
2. **Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene **2**:75-93.
3. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110-2114.
4. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of basic sequence homology in nucleic acids. Methods Enzymol. **21**:413-427.
5. **DeLucia, P., and J. Cairns.** 1969. Isolation of an *E. coli* strain with a mutation affecting DNA polymerase. Nature (London) **274**:1164-1167.
6. **Dougan, G., and D. Sherrat.** 1977. The transposon Tn*1* as a probe for studying ColE1 structure and function. Mol. Gen. Genet. **151**:151-160.
7. **Elwell, L. P., J. M. Inamine, and B. Minshew.** 1978. Common plasmid specifying tobramycin resistance in two enteric bacteria isolated from burn patients. Antimicrob. Agents Chemother. **13**:312-317.
8. **Farrar, W. E., Jr., and N. M. O'Dell.** 1976. β-Lactamases and resistance to penicillins and cephalosporins in *Serratia marcescens*. J. Infect. Dis. **134**:245-251.
9. **Heffron, F., P. Bedinger, J. J. Champoux, and S. Falkow.** 1977. Deletions affecting the transposition of an antibiotic resistance gene. Proc. Natl. Acad. Sci. U.S.A. **74**:702-706.
10. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383**:457-463.
11. **Jacoby, G. A., A. E. Jacob, and R. W. Hedges.** 1976. Recombination between plasmids of incompatibility groups P-1 and P-2. J. Bacteriol. **127**:1278-1285.
12. **Korfhagen, T. R., L. Sutton, and G. A. Jacoby.** 1978. Classification and physical properties of *Pseudomonas* plasmids, p. 221-224. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
13. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.
14. **Mitsuhashi, S., S. Yamagishi, T. Sarvai, and H. Kawabe.** 1977. Biochemical mechanisms of plasmid-mediated resistance, p. 195-251. *In* S. Mitsuhashi (ed.), R-factor, drug resistance plasmid. University Park Press, Tokyo.
15. **Price, K. E., M. D. Defuria, and T. A. Pursiano.** 1976. Amikacin, an aminoglycoside with marked activity against antibiotic resistant clinical isolates. J. Infect. Dis. **S134**:S249-S261.
16. **Rubens, C., F. Heffron, and S. Falkow.** 1976. Transposition of a plasmid deoxyribonucleic acid sequence that mediates ampicillin resistance: independence from host *rec* functions and orientation of insertion. J. Bacteriol. **128**:425-434.
17. **Shahrabadi, M. S., L. E. Bryan, and H. M. Van Den Elzen.** 1975. Further properties of P-2 R factors of *Pseudomonas aeruginosa* and their relationship to other plasmid groups. Can. J. Microbiol. **21**:592-605.
18. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. Methods Enzymol. **43**:737-755.
19. **Smith, D. I., R. G. Lus, M. C. R. Calvo, N. Datta, A. E. Jacob, and R. W. Hedges.** 1975. Third type of plasmid conferring gentamicin resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **8**:227-230.
20. **So, M., F. Heffron, and S. Falkow.** 1978. Method for the genetic labeling of cryptic plasmids. J. Bacteriol. **133**:1520-1523.
21. **Stanisich, V. A., P. M. Bennett, and J. M. Ortiz.** 1976. A molecular analysis of transductional marker rescue analysis involving P-group plasmids in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **143**:333-337.