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Cloning and Expression in *Escherichia coli* of a Gene Encoding Nonenzymatic Chloramphenicol Resistance from *Pseudomonas aeruginosa*

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High-level chloramphenicol resistance in *Pseudomonas aeruginosa* may be due to enzymatic inactivation, ribosomal mutation, or a permeability barrier. We investigated the nonenzymatic resistance mechanism encoded by Tn*1696*, a transposon found in *P. aeruginosa*. A 1-megadalton DNA fragment from Tn*1696* was cloned which mediated expression of chloramphenicol resistance in *Escherichia coli*. Comparison of the effects of chloramphenicol on in vitro translation revealed no difference between the susceptible recipient strain and the resistant transformant containing the cloned gene. The rate of chloramphenicol uptake was slower in the resistant strain, suggesting a permeability barrier to the antibiotic. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membranes demonstrated the absence of a 50,000-dalton protein in the resistant strain. DNA homology was evident between Tn*1696* and chloramphenicol-resistant isolates of *Haemophilus influenzae* possessing altered outer membrane permeability. We conclude that chloramphenicol resistance encoded by Tn*1696* is due to a permeability barrier and hypothesize that the gene from *P. aeruginosa* may share a common ancestral origin with these genes from other gram-negative organisms.

Chloramphenicol resistance in gram-negative bacteria is most frequently due to the production of an inactivating enzyme, chloramphenicol acetyltransferase (12). Alternative resistance mechanisms that have been described include decreased ribosomal sensitivity to the action of chloramphenicol (28) and decreased penetration of the drug into bacterial cells (8, 14, 23).

In both *Pseudomonas aeruginosa* and *Escherichia coli*, a plasmid-mediated permeability barrier to chloramphenicol has been reported (14, 20), although the nature of the barrier has not been defined. Chromosomally encoded resistance due to alterations of outer membrane permeability and a deficiency of porin proteins has been reported in certain members of the family *Enterobacteriaceae* (10, 13, 24) and in *Haemophilus influenzae* (8).

Rubens et al. (31) have reported a transposon from a plasmid found in *P. aeruginosa* (Tn*1696*) which mediates nonenzymatic chloramphenicol resistance and resistance to four other antimicrobial agents. A 1-megadalton (MDa) DNA fragment was cloned from Tn*1696* which mediates expression of chloramphenicol resistance in *E. coli*. This enabled the characterization of the chloramphenicol resistance mechanisms encoded by Tn*1696*; ribosomal susceptibility and antibiotic penetration were examined in *E. coli* that expressed the resistance gene. In addition, hybridization experiments were performed to determine DNA homology between the Tn*1696* gene and a chromosomal chloramphenicol resistance gene recently described in *H. influenzae* (8).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* C600(pCER100) was the source of purified pCER100 plasmid DNA (31). *E. coli* HB101(pBR322) and JM83(pACYC184) (5) were the sources of transforming plasmids. *E. coli* C600 (3) and HB101 (5) were used as recipients in transformation.

H. influenzae 76-81739 is a nontypable chloramphenicol-resistant clinical isolate which was obtained from C. Thornsberry (27), and strain MAP is a well-characterized laboratory strain (9). TF 76-81739-7 is a chloramphenicol-resistant strain constructed by transformation of strain MAP with donor DNA from strain 76-81739 (8).

Tn*1696* is a 9.1-MDa transposon that resides within R1033, a 45-MDa plasmid of the P-1 incompatibility group of *P. aeruginosa*; Tn*1696* mediates resistance to chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, and mercuric chloride (31). The ColE1 derivative plasmid pMB8 is 1.72 MDa and mediates immunity to colicin E1 protein (6). Plasmid pCER100 is a 10.8-MDa composite formed between pMB8 and Tn*1696* (31). Plasmid pBR322 is a 3.2-MDa nonconjugative plasmid that mediates resistance to ampicillin and tetracycline (5).

Plasmid pACYC184 is a 2.7-MDa nonconjugative plasmid which encodes for the production of chloramphenicol acetyltransferase and confers resistance to chloramphenicol and tetracycline (5).

Media. Liquid medium was L broth (22) for *E. coli* strains and supplemented brain heart infusion broth (8) for *H. influenzae* strains. Broth cultures were incubated at 37°C and shaken at 200 cycles per min. Solid medium was L agar for *E. coli* strains and supplemented brain heart infusion agar for *H. influenzae* strains. Plate cultures were incubated at 37°C, and *H. influenzae* strains were incubated with 5% CO_2.

DNA preparation and analysis. Plasmid DNA was purified from cleared lysates of *E. coli* by cesium chloride-ethidium bromide ultracentrifugation (22). Chromosomal DNA from *H. influenzae* was isolated by using a modification of the technique of Hull et al. (17). Restriction endonuclease digestions were carried out as described by Maniatis et al. (22). DNA was analyzed by 0.7% agarose horizontal slab gel electrophoresis in Tris borate buffer (22). Individual restric-

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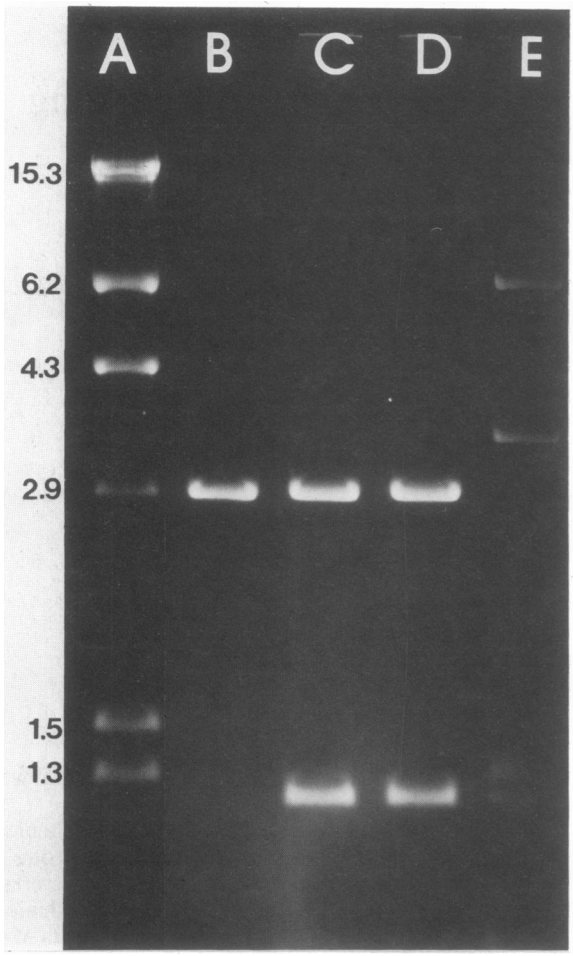

FIG. 1. Agarose gel electrophoresis of *Hin*dIII fragments of bacteriophage lambda (lane A), pBR322 (lane B), pFYB155 (lane C), pFYB189 (lane D), and pCER100 (lane E). Molecular weights (×10,000) are listed on the left margin. Plasmids pFYB155 and pFYB189 are composites formed between pCER100 and pBR322 and selected on chloramphenicol-containing media. Plasmid pFYB155 is ampicillin and chloramphenicol resistant; pFYB189 is ampicillin, chloramphenicol, and mercuric chloride resistant.

tion fragments were isolated by electroelution from agarose gels or 5% sodium dodecyl sulfate-polyacrylamide gels (22). The method of Smith and Summers (33) was used to bidirectionally transfer DNA from agarose gels to nitrocellulose filters. Hybridization of filters to ^{32}P-labeled DNA was performed by the method of Wahl et al. (37). Radiolabeled DNA was prepared by in vitro nick translation (29).

Cloning methodologies. Purified pCER100 and pBR322 were digested to completion with *Hin*dIII, mixed at a ratio of 3:1, and ligated with T_4 DNA ligase for 24 h (22). Transformation of $CaCl_2$-treated *E. coli* HB101 was performed by the method of Cohen et al. (11), and transformants were selected on MacConkey agar media containing 50 μg of ampicillin and 15 μg of chloramphenicol per ml. After overnight incubation, transformant colonies were picked for further characterization.

MIC. Determination of the MIC of chloramphenicol was performed by standard techniques with an inoculum of 10^5 CFU. MICs were determined by twofold broth dilution for *E. coli* strains (35) and by agar dilution with a Steers replicator for *H. influenzae* strains (34).

In vitro translation. Ether-treated cells (8) were used to examine the effect of increasing chloramphenicol concentration on translation of endogenous mRNA. Chloramphenicol concentrations ranged from 0 to 500 μg/ml.

Assay for chloramphenicol acetyltransferase. Cell sonic extracts were prepared as described previously (8), and chloramphenicol acetyltransferase activity was assayed by the spectrophotometric technique of Shaw and Brodsky (32).

Chloramphenicol permeability. The penetration of chloramphenicol into whole bacterial cells was determined by a modification of the technique of Gaffney et al. (14). High-pressure liquid chromatography was used to quantitate the chloramphenicol concentration remaining in culture media following overnight incubation (8).

Isolation of outer membrane proteins. Outer membrane proteins of *E. coli* strains were isolated by detergent solubilization as described by Achtman et al. (1). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% acrylamide gel by the method of Laemmli (21). Proteins were visualized by Coomassie blue staining (16).

RESULTS

Cloning the Tn*1696* chloramphenicol resistance gene in *E. coli*. Digestion of pCER100 with *Hin*dIII resulted in 6.2-, 3.4-, 1.2-, and 1.0-MDa fragments (Fig. 1). When these fragments were ligated with pBR322 and transformed into HB101, the 1.0-MDa fragment was found to encode the chloramphenicol resistance gene. This fragment may also encode part or all of the mercury resistance gene as well, because both ampicillin- and chloramphenicol-resistant clones and ampicillin-, chloramphenicol-, and mercuric chloride-resistant clones containing this fragment were isolated. One clone, HB101(pFYB155), which is ampicillin and chloramphenicol resistant only, was chosen for further analysis.

MICs. The MIC of chloramphenicol for the *E. coli* and *H. influenzae* strains are listed in Table 1. Strains HB101(pFYB155) and 76-81739 had similar levels of resistance. The MICs were lower than those seen in chloramphenicol acetyltransferase-producing strains of *E. coli* (12). Serial passage of HB101(pFYB155) on agar containing increasing concentrations of chloramphenicol did not result in an increased MIC.

In vitro translation. The effect of chloramphenicol on ribosomal protein synthesis in ether-extracted cells of HB101 and HB101(pFYB155) is shown in Fig. 2. With chloramphenicol concentrations from 0 to 500 μg/ml, there was no significant difference between the strains in the inhibitory effect of the antibiotic on amino acid incorporation.

TABLE 1. MICs of chloramphenicol

Strain	MIC (μg/ml)[a]
E. coli[b]	
HB101	3.125
HB101(pFYB155)	100
H. influenzae[c]	
MAP	0.1
76–81739	50
TF 76–81739–7	10

[a] Inoculation of 10^5 CFU.
[b] MIC was determined by broth dilution.
[c] MIC was determined by agar dilution.

Chloramphenicol permeability. The penetration of chloramphenicol into HB101 was difficult to determine because concentrations that were high enough to assay were bactericidal to the organism during the overnight uptake period. Introduction of chloramphenicol acetyltransferase activity into the chloramphenicol-susceptible strain HB101 enabled the use of antibiotic concentrations that were more than 10 times the MIC. Plasmid pACYC184 was used to transform both strains to chloramphenicol acetyltransferase production. This allowed the comparison of chloramphenicol loss from culture supernatants following overnight incubation using an initial concentration of 50 μg/ml. The results of permeability experiments are shown in Table 2. The rate of loss was similar in the first hour of incubation, perhaps due to nonspecific binding. However, after overnight incubation, the culture medium of HB101(pFYB155, pACYC184) had significantly less antibiotic loss compared with that of HB101(pACYC184). This difference was not due to differential drug inactivation because chloramphenicol acetyltransferase activity in the two strains was comparable: 2.48 U/mg of protein in HB101(pACYC184) and 2.78 U/mg of protein in HB101(pACYC184, pFYB155).

Outer membrane protein profile. The outer membrane protein profiles of HB101, HB101(pBR322), and HB101(pFYB155) are shown in Fig. 3. HB101 and HB101(pBR322) contained an outer membrane protein with an apparent molecular weight of 50,000 which was not visualized in HB101(pFYB155). In addition, HB101 demonstrated an outer membrane protein with an apparent molecular weight

TABLE 2. Chloramphenicol concentration in culture supernatant

Strain	Incubation time (h)	Chloramphenicol concn (μg/ml)
HB101(pACYC184)	0	51.7
	20	7.9[a]
HB101(pFYB155, pACYC184)	0	46.0
	20	33.4[a]

[a] $P < 0.005$, using the one-tailed Student t test.

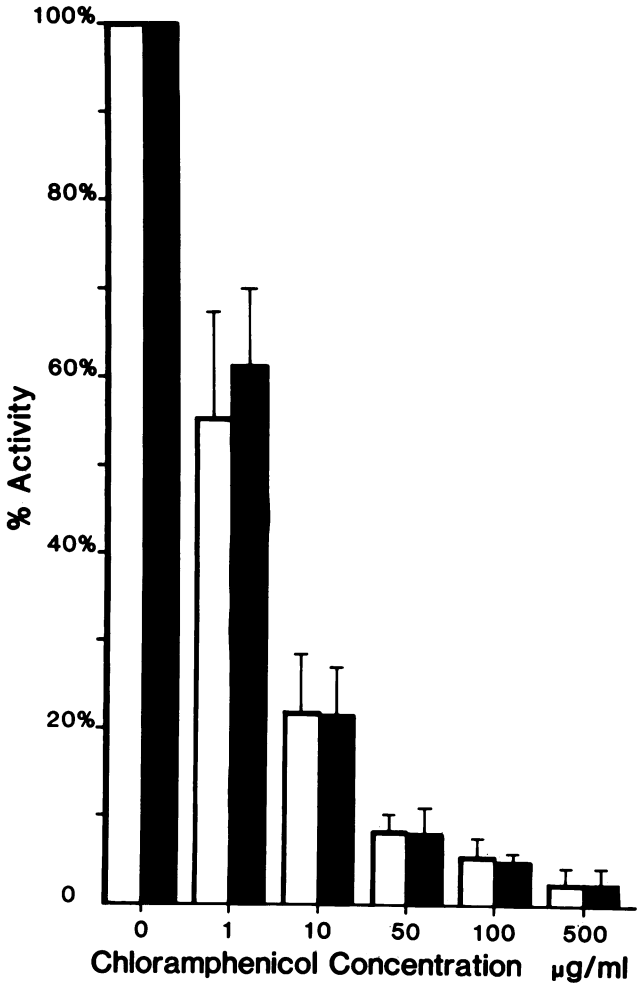

FIG. 2. The effect of chloramphenicol (concentration, 0 to 500 μg/ml) on in vitro translation (incorporation of [^{14}C]valine into trichloroacetic acid-precipitable material) in ether-extracted cells of HB101 (■) and HB101(pFYB155) (□).

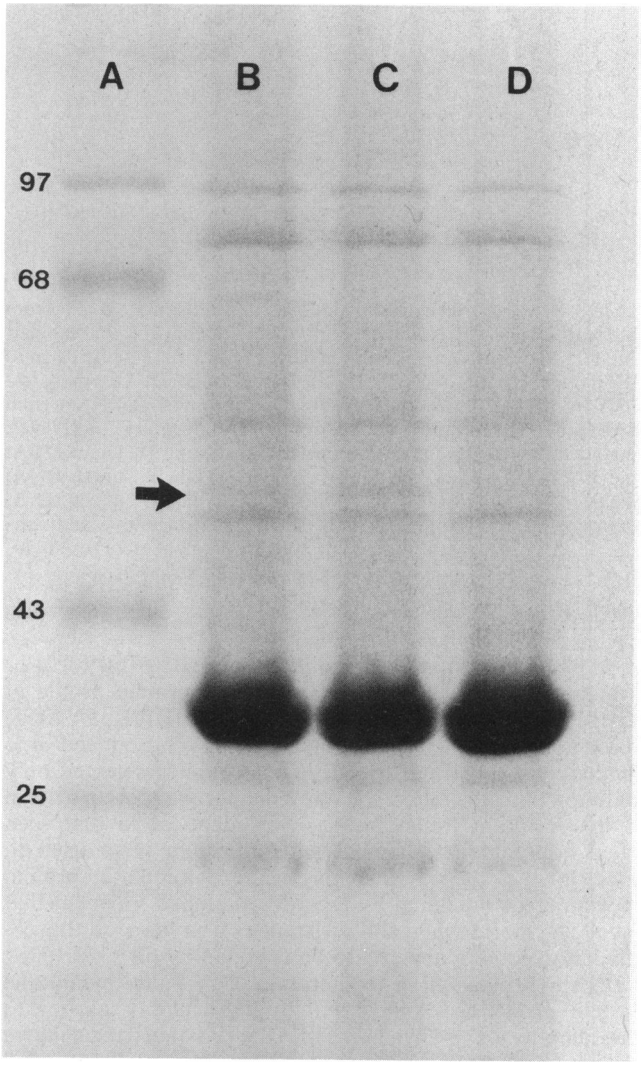

FIG. 3. Outer membrane proteins of HB101, HB101(pBR322), and HB101(pFYB155) extracted by the technique of Achtman et al. (1). Lane A, Molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.); lane B, HB101; lane C, HB101(pBR322); lane D, HB101(pFYB155). The arrow designates the 50,000-dalton protein that is visualized in HB101 and HB101(pBR322) but that is missing from HB101(pFYB155).

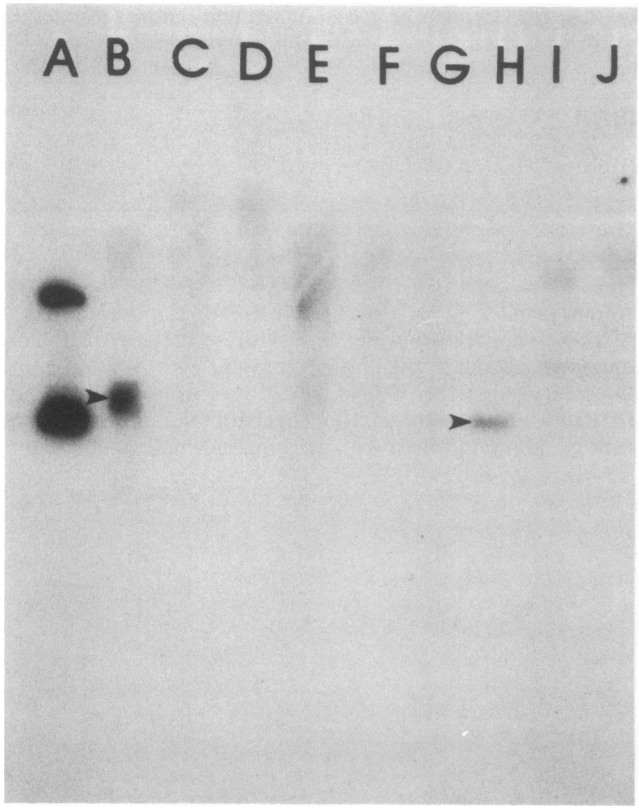

FIG. 4. Southern hybridization of chromosomal DNA from chloramphenicol-resistant *H. influenzae* using the ^{32}P-labeled 1.0-MDa *Hin*dIII fragment purified from pFYB155. Lane A, Plasmid DNA from HB101(pFYB155); lanes B through D, chromosomal DNA from 76-81739; lanes E through G, chromosomal DNA from MAP; lanes H through J, chromosomal DNA from TF 76-81739-7; lane B, *Eco*RI digest; lane C, *Bam*HI digest; lane D, uncut DNA; lane E, *Eco*RI digest; lane F, *Bam*HI digest; lane G, uncut DNA; lane H, *Eco*RI digest; lane I, *Bam*HI digest; lane J, uncut DNA. Apparent DNA homology is seen under midstringency conditions with an *Eco*RI fragment (arrow) of both chloramphenicol resistance strains; no specific homology is apparent with strain MAP.

of 68,000 which was not seen in either HB101(pBR322) or HB101(pFYB155). The outer membrane protein profile of HB101(pFYB189) was identical to that of HB101(pFYB155) (data not shown). When pFYB155 was introduced into competent HB101 and transformant colonies selected only for ampicillin resistance, the outer membrane protein pattern of the resultant transformants was identical to that seen when both chloramphenicol and ampicillin were used to select for the colonies. The loss of the 50-kilodalton protein appears to be mediated by the 1-MDa cloned fragment that encodes chloramphenicol resistance. The loss of the 68-kilodalton protein appears to be encoded by pBR322.

DNA hybridization. The alteration of the outer membrane protein profile associated with a permeability barrier to chloramphenicol is suggestive that the resistance mechanism encoded by Tn*1696* is similar to the chromosomally encoded resistance reported in *H. influenzae* (8). To test this hypothesis, the 1.0-MDa *Hin*dIII fragment from the cloned insert was purified from pFYB155 and hybridized against chromosomal DNA isolated from chloramphenicol-resistant *H. influenzae* 76-81739 and TF 76-81739-7 and the susceptible strain MAP. The results of hybridization experiments are shown in Fig. 4. Under midstringency conditions (25% formamide; wash temperature, 50°C), there was apparent DNA homology between the 1.0-MDa fragment from Tn*1696* and restriction fragments of chromosomal DNA from the resistant *H. influenzae* strains, but not strain MAP.

DISCUSSION

Chloramphenicol resistance is frequently encountered in both gram-positive and gram-negative bacteria. Reported resistance mechanisms include antibiotic inactivation, ribosomal resistance, and altered bacterial permeability.

Enzymatic inactivation catalyzed by chloramphenicol acetyltransferase is the most common mechanism of resistance described in *Staphylococcus aureus*, *H. influenzae*, and members of the family *Enterobacteriaceae* (12, 30, 32). Rubens et al. (31) have previously reported that the resistance mechanism encoded by Tn*1696* (a transposon isolated from a resistance plasmid found in *Pseudomonas*) is not due to the production of chloramphenicol acetyltransferase. In addition, incubation of strain HB101(pFYB155) with 20 μg of chloramphenicol per ml and assay of the supernatant by high-pressure liquid chromatography revealed no alteration of the antibiotic (data not shown).

Decreased ribosomal susceptibility to the action of chloramphenicol is a second potential resistance mechanism. Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit (26), and alteration of this target would be expected to result in resistance. Although there are several reports of chloramphenicol resistance genes that map in the ribosomal protein region of the chromosome (2, 4) in *E. coli* and *Bacillus subtilis*, Osawa et al. (28) are the only researchers to have described alterations in the binding of chloramphenicol to the 50S subunit in *B. subtilis*. Utilizing ether-extracted bacterial cells, we could not demonstrate that Tn*1696* encodes ribosomal resistance to chloramphenicol.

Altered outer membrane permeability is a third mechanism of resistance that has been reported. Decreased permeability to chloramphenicol has been described in members of the family *Enterobacteriaceae*, *H. influenzae*, and *P. aeruginosa* (8, 14, 20). In both *E. coli* and *H. influenzae*, permeability changes have been associated with the loss of specific outer membrane proteins. The *cmlB* mutant of *E. coli* K-12 lacks a 37,000-dalton protein which functions as a porin for the transport of nutrients as well as antibiotics (10, 13, 24). We recently described four strains of chloramphenicol-resistant *H. influenzae* which lack a 40,000-dalton outer membrane protein (8). This outer membrane protein appears to function as a porin in *H. influenzae* (36; J. L. Burns and A. L. Smith, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 992, 1985).

A permeability barrier to chloramphenicol is a common resistance mechanism in *P. aeruginosa*. Mitsuhashi et al. (23) found that up to 50% of the plasmids that encode chloramphenicol resistance conferred altered antibiotic uptake. The character of that barrier has not been defined, although some data suggest that it may be porin mediated as well. Irvin and Ingram (18) have reported chloramphenicol-resistant strains of *P. aeruginosa* which have reduced ability to accumulate amino acids. Porin-deficient strains of *P. aeruginosa* have been found to have decreased permeability to β-lactam antibiotics and aminoglycosides (7, 15, 25, 38); however, the role of porins in the entry of chloramphenicol has not been examined in *P. aeruginosa*.

Iyobe et al. (19) reported transposon-encoded chloramphenicol resistance from the P-2 plasmid Rm3159-1 in *P.*

aeruginosa which did not result from the production of chloramphenicol acetyltransferase. They isolated the transposon (Tn*2001*) and localized the resistance determinant to a 2.1-kilobase DNA fragment.

We have reported here the cloning of a transposon-mediated resistance gene isolated from a *P. aeruginosa* plasmid which encodes for the expression in *E. coli* of a permeability barrier to chloramphenicol. This was associated with an alteration in the outer membrane protein profile: the loss of a 50,000-dalton protein. These findings suggest that the resistance mechanism encoded by Tn*1696* may be similar to that found in permeability mutants of *H. influenzae*; both resistance genes mediated decreased antibiotic penetration associated with the loss of outer membrane proteins. Results of DNA hybridization experiments, using the cloned *P. aeruginosa* gene as a probe for homology with chromosomal DNA from chloramphenicol-resistant *H. influenzae*, suggest that there is a genetic relatedness of the resistance mechanisms in these organisms. This homology occurred at midstringency, and the 1-MDa fragment may contain homologous DNA sequences unrelated to chloramphenicol resistance. Whether the chloramphenicol resistance gene from Tn*1696* shares DNA sequence homology with these *H. influenzae* strains or with Tn*2001* has not been determined.

We conclude that the mechanism of chloramphenicol resistance encoded by Tn*1696* is a permeability barrier, possibly due to the loss of a porin from the outer membrane. A moderate degree of DNA homology with porin-deficient strains of *H. influenzae* supports the concept of a common ancestral origin of chloramphenicol resistance genes. Further characterization of the Tn*1696* chloramphenicol resistance gene will include the isolation of a smaller DNA fragment from pFYB155, the determination of its sequence relatedness to *H. influenzae* and *E. coli* with altered permeability, and eventual nucleotide sequence analysis.

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