ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1982, p. 358–363
0066-4804/82/090358-06$02.00/0

Vol. 22, No. 3

Plasmid-Mediated Gentamicin Resistance of *Pseudomonas aeruginosa* and Its Lack of Expression in *Escherichia coli*

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Received 8 February 1982/Accepted 23 June 1982

We isolated 11 nonconjugative plasmids mediating resistance to aminoglycoside antibiotics, including gentamicin, from *Pseudomonas aeruginosa* strains. Their genetic properties were investigated in both *P. aeruginosa* and *Escherichia coli* transformants. The plasmid molecular weights ranged from 11×10^6 to 24×10^6. A low level or complete absence of gentamicin resistance was observed when these plasmids were introduced into *E. coli*, but gentamicin resistance was restored when the plasmids were transferred back to *P. aeruginosa* from *E. coli*. Aminoglycoside-modifying enzyme activity was detected in *P. aeruginosa* harboring these plasmids, but was absent or greatly reduced in *E. coli* strains. This lack of expression may explain the observed decrease in aminoglycoside resistance.

Plasmids isolated from *Pseudomonas aeruginosa* have been classified into 11 incompatibility groups. Most of the conjugative plasmids are nontransmissible to *Escherichia coli* strains, except for plasmids of the P-1 or P-3 group (12). Three reasons for this nontransferability to *E. coli* can be postulated: (i) nontransmissibility owing to some differences in cell surface structure; (ii) no replication of the plasmids after transmission to *E. coli* strains; and (iii) failure of resistance gene expression in the *E. coli* system. Some of the plasmids mobilized to *E. coli* by the presence of P-1 group plasmids can replicate by themselves in that host (12, 13, 16). In contrast, many plasmids are incapable of replication in *E. coli*, except by recombination with the mobilizing plasmid (13, 20).

Recently the isolation frequency of gentamicin-resistant *P. aeruginosa* strains has increased (3, 4, 14, 18, 21, 24, 26). We have also isolated plasmids mediating gentamicin resistance and transferable by conjugation to a recipient *P. aeruginosa* strain in 20% of the 91 gentamicin-resistant *P. aeruginosa* isolates from clinical specimens in Japan. Gentamicin-resistance plasmids which were transfer deficient were also found in 56% of the gentamicin-resistant strains. In this report, we examined the genetic properties of such nonconjugative plasmids mediating gentamicin resistance in *P. aeruginosa* and also in *E. coli* after being introduced by transformation.

MATERIALS AND METHODS

Media. Heart infusion agar (Eiken Kagaku Co., Tokyo, Japan) and peptone water were used for the determination of drug resistance. Peptone water consisted of 1,000 ml of distilled water, 5 g of NaCl, and 10 g of peptone. For the assay of sulfanilamide resistance, A-glucose agar or semisynthetic medium was used. A-glucose agar consisted of medium A (6) without sodium citrate but contained 0.008% bromothymol blue, 1.5% agar, and 0.1% glucose. Semisynthetic medium consisted of 1,000 ml of medium A, 2 g of Casamino Acids, 10 mg of tryptophane, 2 mg of nicotinic acid, 10 mg of thiamine, 2 g of glucose, and 15 g of agar. BTB agar used for the selection of transformants and transconjugants consisted of nutrient broth (10 g of beef extract, 10 g of peptone, and 3 g of NaCl in 1,000 ml of distilled water) supplemented with 0.008% bromothymol blue and 1.5% agar. L-broth (22) was used for the preparation of competent cells for transformation. Penassay broth (Difco Laboratories, Detroit, Mich.) was used as the medium for conjugation. Medium B consisted of 10 g of peptone, 2 g of yeast extract, 8 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 1.2 g of $(NH_4)_2SO_4$, 2 g of glucose, and 0.4 g of $MgSO_4$ in 1,000 ml of distilled water.

Bacterial strains. A total of 91 gentamicin-resistant strains of *P. aeruginosa* were isolated from clinical specimens at 12 hospitals geographically dispersed in Japan. *P. aeruginosa* ML5019 (rifampin-resistant mutant of ML5018), ML5066 (nalidixic acid-resistant mutant of PAO2142), ML4561 (rifampin-resistant mutant of Ps1008), and *E. coli* ML4905 (rifampin-resistant mutant of *X*1037) were used as the recipients for plasmids. ML5018 was a Rec^- mutant of PAO2142 (17). Both PAO2142 and Ps1008 ($Leu^-Arg^-Ilv^-His^-Trp^-$) were gifts from H. Matsumoto (Shinshu University, Matsumoto, Japan), whereas *E. coli* K12 *X*1037 (17) was obtained from H. Yoshikawa (Institute of Medical Science, University of Tokyo, Japan).

Conjugation. Conjugation was carried out in broth (15) or on a membrane filter (17).

Transformation. Transformation was carried out with 0.1 M $MgCl_2$-treated *P. aeruginosa* (23, 25) or with 30 mM $CaCl_2$-treated *E. coli* (5). Competent cells were prepared by using early log-phase cells of *P.*

aeruginosa and *E. coli* in L-broth. After harvest, the cells were washed once with one-half of the original volume of 0.1 M $MgCl_2$ for *P. aeruginosa* or 30 mM $CaCl_2$ for *E. coli*. The cells were centrifuged and resuspended in 0.5 volume of the same solution described above and kept at 4°C for 20 min. After centrifugation, the cells were resuspended in 0.1 volume of Mg^{2+}-solution for *P. aeruginosa* or 0.5 volume of Ca^{2+}-solution for *E. coli*. The drug concentrations of gentamicin (in micrograms per milliliter) contained in BTB agar used for the selection of transformants in *P. aeruginosa* were: 0.8 for ML5019 and 6.3 for ML4561. The drug concentrations used for the selection of transformants in *E. coli* ML4905 were: gentamicin, 0.8; kanamycin, 12.5; streptomycin, 6.3; mercuric chloride, 12.5; and carbenicillin, 50.

Preparation of cleared lysate and isolation of plasmid DNA. Plasmid DNA was purified from cleared lysates by ultracentrifugation in ethidium bromide-cesium chloride gradients (17). Alternatively, a small-scale purification technique (2) was used. The cleared lysate was prepared by sodium dodecyl sulfate-alkaline treatment, and plasmid DNA was precipitated with ethanol after removal of chromosomal DNA fragments.

Agarose gel electrophoresis. Plasmid DNA was subjected to electrophoresis at 100 mA for 1.5 h, using horizontal 0.6% agarose gels in TEAS buffer (pH 8.0) containing 50 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 18 mM NaCl. After the DNA was stained with ethidium bromide, the gels were photographed with UV light.

Estimation of molecular weight. Plasmid DNAs were visualized under an electron microscope according to the method of Yamagishi et al. (27). The double-stranded replicative form of fd DNA with a size of 6.4 kilobase pairs was used as the internal standard in electron microscopic observations (1). The molecular weight of plasmid DNA was estimated as the mean value of about 20 molecules.

Assay of aminoglycoside-modifying enzymes. Preparation of cell-free extract and assay of aminoglycoside-modifying enzymes were carried out by the slightly modified procedure described previously (24). For the bioassay of enzymes, 0.15 ml of S-30 fraction was supplied to the mixture containing 0.05 ml of coenzyme (20 mM ATP or 1 mM acetyl coenzyme A), 0.05 ml of 20 mM $MgCl_2$, and 0.2 ml of 0.2 M buffer (sodium acetate, pH 6; Tris-malate, pH 7; Tris-hydrochloride, pH 8). The reaction mixture was incubated at 37°C for 6 h and then stopped by heating at 100°C for 1 min. The residual activity of antibiotics in the reaction mixture was determined by the cup method with *Bacillus subtilis* ATCC 6633 as a test organism. Radioisotope assay was carried out with [1-^{14}C]acetyl coenzyme A (10 μCi/ml), [γ-^{32}P]ATP (50 μCi/ml), or [8-^{14}C]ATP (10 μCi/ml). The reaction mixture consisted of 30 μl of S-105 fraction (10 mg of protein per ml), 10 μl of 20 mM $MgCl_2$, 10 μl of 1 mM drug, and 10 μl of coenzyme. The reaction was carried out at 37°C for 2 h.

RESULTS

Isolation of plasmids mediating gentamicin resistance. We could isolate conjugative plasmids mediating gentamicin resistance from 22 of 91 gentamicin-resistant *P. aeruginosa* strains. We selected 14 gentamicin-resistant strains from the remaining 69 strains without conjugative plasmids owing to differences in their resistance patterns, clinical sources, or both. We isolated plasmid DNA from these 14 strains and transferred the DNA to *P. aeruginosa* ML5019 by transformation. We obtained 11 nonconjugative plasmids mediating gentamicin resistance from nine strains and none from the remaining five strains. Seven of these plasmids were randomly selected for further studies. Their resistance patterns and molecular weights are shown in Table 1.

We transposed Tn*1* (9) to pMS101 and pMS105 for the selection of these plasmids in transformation experiments. Plasmid DNAs were prepared from ML5019 carrying a donor plasmid with Tn*1* and pMS101 or pMS105. Transformation of ML5019 was carried out, and Tn*1* transposon derivatives were selected on BTB plates containing both carbenicillin and gentamicin. The transposon derivatives, i.e., pMS101::Tn*1* and pMS105::Tn*1*, were called pMS112 and pMS113.

All plasmids mediated resistance to dibekacin, gentamicin, kanamycin, netilmicin, sagamicin, sisomicin, and tobramycin. Phenotypic expression of resistance to amikacin, fortimicin, and lividomycin was not observed.

The conjugal transferability of seven plasmids from the ML5019 transformants to an isogenic strain, ML5066, was examined. None of the seven plasmids showed conjugal transferability by either mixed culture in broth or the membrane filter method.

Transformation with nonconjugative plasmids. Plasmid DNAs isolated from *P. aeruginosa* ML5019 transformants were transformed to both *P. aeruginosa* ML4561 and *E. coli* ML4905. Transformants of *E. coli* ML4905 were selected on plates containing each of the antibiotics to which the plasmids provided resistance. *E. coli* transformants with plasmid DNAs were

TABLE 1. Isolation of nonconjugative plasmids from *P. aeruginosa* strains

Plasmid	Resistance pattern[a]	Mol wt (×10^6)
pMS106	Gm Hg Km Sm Su	22
pMS107	Gm Hg Km Sm Su	22
pMS108	Gm Hg Km Sm Su	24
pMS109	Cm Gm Hg Km Sm Su	23
pMS111	Gm Hg Km Sm Su	22
pMS101	Gm Km Su	14
pMS112	Gm Km Su::Tn*1*	17
pMS105	Cm Gm Km Sm Su	11
pMS113	Cm Gm Km Sm Su::Tn*1*	14

[a] Abbreviations: Cm, chloramphenicol; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Sm, streptomycin; Su, sulfanilamide.

obtained on selection plates containing carbenicillin or mercuric chloride but not on plates containing gentamicin, kanamycin, streptomycin, or chloramphenicol. On the other hand, transformants of *P. aeruginosa* ML4561 could be selected by all drugs to which resistance was mediated by each of the plasmids.

Levels of resistance mediated by gentamicin resistance plasmids in *P. aeruginosa* and *E. coli* strains. Resistance levels of *P. aeruginosa* and *E. coli* bearing the seven plasmids are shown in Table 2. The minimal inhibitory concentration (MIC) level of gentamicin was markedly decreased in *E. coli* transformants; gentamicin resistance mediated by six plasmids (pMS106-109, -111, and -113) was not detected. The levels of resistance to kanamycin, streptomycin, or chloramphenicol were greatly decreased. Thus, the level of the plasmid-mediated resistance to aminoglycoside antibiotics was greatly decreased in *E. coli* relative to the same plasmid in a *P. aeruginosa* host. The level of resistance to other drugs (carbenicillin and mercuric chloride) in *E. coli* was the same as those in a *P. aeruginosa* host.

Next, plasmid DNA was isolated from each of the *E. coli* transformants and introduced back to *P. aeruginosa* ML4561 by transformation. Transformants of *P. aeruginosa* ML4561 were obtained with all seven plasmids, and their resistance levels were the same as those of *P. aeruginosa* ML4561 which possessed the original plasmids (Table 2).

When plasmid DNA was isolated from either *P. aeruginosa* or *E. coli* transformants and visualized by agarose gel electrophoresis, the molecular weight of each plasmid was found to be the same in both bacteria (Fig. 1). The restriction pattern of each plasmid by restriction enzyme *Eco*RI was also the same in both bacterial hosts.

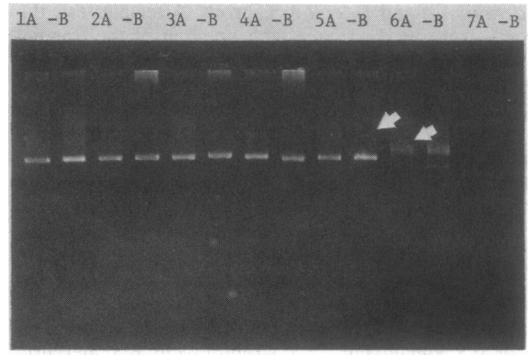

FIG. 1. Agarose gel electrophoresis of gentamicin resistance plasmids. Lane 1, pMS106; lane 2, pMS107; lane 3, pMS108; lane 4, pMS109; lane 5, pMS111; lane 6, pMS112; and lane 7, pMS113. Plasmid DNAs were isolated from either *P. aeruginosa* ML5019 (A) or *E. coli* ML4905 (B) transformants. Arrows, Open circular form of plasmid DNA.

Assay of aminoglycoside-modifying enzymes. Activities of aminoglycoside-modifying enzymes were determined by using the extracts of plasmid-bearing *P. aeruginosa* ML5019 and plasmid-bearing *E. coli* ML4905. At least two enzymes were found in *P. aeruginosa* transformants by bioassay, and the substrate profile of enzymes is shown in Table 3. In contrast, no enzyme activity could be found in *E. coli* transformants by bioassay.

The results of the incorporation of [^{14}C]acetyl coenzyme A into gentamicin C_{1a} or kanamycin A are also shown in Table 3. Activity of acetyltransferase in *E. coli* extract was less than 9% of that in *P. aeruginosa* extract. The incorporation of [γ-^{32}P]ATP or [^{14}C]ATP into gentamicin C_{1a} or kanamycin A was also determined. When

TABLE 2. Levels of resistance mediated by plasmids in two host bacteria

Plasmid	Levels of resistance[a] to:													
	Cb		Cm		Gm		Hg		Km		Sm		Su	
	P[b]	E[c]	P	E	P	E	P	E	P	E	P	E	P	E
pMS106	50	3.2	12.5	3.2	25	0.2 (125)[d]	100	50	100	3.2	>800	6.3	>6,400	50
pMS107	50	3.2	25	3.2	25	0.2 (125)	100	50	100	3.2	>800	6.3	>6,400	50
pMS108	50	1.6	25	6.3	50	0.2 (250)	100	50	200	6.3	>800	6.3	>6,400	100
pMS109	12.5	3.2	200	12.5	25	0.2 (125)	100	50	200	12.5	>800	6.3	>6,400	100
pMS111	50	3.2	12.5	3.2	25	0.2 (125)	100	50	800	3.2	>800	6.3	>6,400	100
pMS112	>800	>800	12.5	3.2	100	0.8 (125)	12.5	3.2	200	100	12.5	0.8	>6,400	400
pMS113	>800	>800	100	3.2	25	0.1 (250)	12.5	3.2	100	3.2	800	0.8	>6,400	3.2
—[e]	50	3.2	12.5	3.2	1.6	0.2 (8)	12.5	3.2	25	3.2	12.5	0.8	400	0.8

[a] Levels of resistance are expressed as the MIC (micrograms per milliliter). For abbreviations, see Table 1, footnote *a*.

[b] P, *P. aeruginosa* ML4561.

[c] E, *E. coli* ML4905.

[d] Number in parentheses indicates the ratio of the MIC levels of gentamicin in *P. aeruginosa* to those in *E. coli*.

[e] —, Host bacteria without plasmid.

TABLE 3. Detection of aminoglycoside-modifying enzymes in two host bacteria

Host	Plasmid	Enzyme activity[a]															
		Acetyltransferase								Phosphotransferase or adenylyltransferase							
		GmC_{1a}[b]	GmC_1	KmA[b]	KmB	Ss	Nt	Dkb	Lv	GmC_{1a}	GmC_1	KmA	KmB	Ss	Nt	Dkb	Lv
P. aeruginosa ML5019	pMS106	+(1,618)	−	+(4,240)	+	+	+	+	−	−	+	+	+	−	−	+	−
	pMS107	+(1,146)	−	+(2,098)	−	+	+	+	−	+	−	+	−	+	−	+	−
	pMS108	+(1,617)	−	+(3,829)	+	+	+	+	−	−	−	+	+	+	−	−	−
	pMS109	+(2,840)	−	+(8,917)	+	+	+	+	−	+	+	+	+	+	−	+	−
	pMS111	+(1,275)	−	+(3,268)	+	+	+	+	−	−	−	−	−	−	+	−	−
	pMS112	+(6,276)	−	+(11,945)	+	+	+	+	−	+	−	+	+	+	−	+	−
	pMS113	+(1,897)	+	+(2,509)	+	+	+	+	−	+	−	−	+	+	−	+	−
	—[c]	−(0)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−
E. coli ML4905	pMS106	−(83)	−	−(187)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS107	−(0)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS108	−(43)	−	−(335)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS109	−(0)	−	−(223)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS111	−(0)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS112	−(53)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−
	pMS113	−(0)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−
	—[c]	−(0)	−	−(0)	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] +, Enzyme activity was detected; −, enzyme activity was not detected.

[b] The incorporation of [^{14}C]acetyl coenzyme A into GmC_{1a} or KmA is expressed in cpm in parentheses. Abbreviations: GmC_{1a}, gentamicin C_{1a}; GmC_1, gentamicin C_1; KmA, kanamycin A; KmB, kanamycin B; Ss, sisomicin; Nt, netilmicin; Dkb, dibekacin; and Lv, lividomycin.

[c] —, Host bacteria without plasmid.

extracts of plasmid-bearing *P. aeruginosa* were used, [γ-^{32}P]ATP was incorporated into both drugs at a low level but [^{14}C]ATP was not. Incorporation of [^{32}P]ATP or [^{14}C]ATP was not observed using the extracts of plasmid-bearing *E. coli*.

DISCUSSION

We isolated nonconjugative plasmids mediating resistance to gentamicin and other drugs. Introducing these plasmids to *E. coli* strains by transformation, they could replicate stably, but their gentamicin resistance was hardly detectable, whereas resistance to carbenicillin or mercuric chloride was normally expressed in the *E. coli* strains. However, the level of gentamicin resistance was restored to the same level as in the original *P. aeruginosa* strains when the plasmids were transferred back to *P. aeruginosa* from *E. coli* transformants. The molecular weight of each plasmid was found to be the same in both bacteria. These results indicated that deletion of plasmid DNA did not occur in *E. coli* after transformation from *P. aeruginosa*. Similarly, deletion of plasmid DNA did not take place after transformation back from *E. coli* transformants to *P. aeruginosa*.

These plasmids conferred upon *P. aeruginosa* resistance to aminoglycoside antibiotics such as kanamycin, dibekacin, netilmicin, tobramycin, and sisomicin in addition to gentamicin, but did not mediate amikacin, fortimicin, and lividomycin resistance. We detected at least two aminoglycoside-modifying enzymes in *P. aeruginosa* transformants, but hardly any enzyme activity could be detected in *E. coli* transformants. In the latter case, the absence of enzyme activity was paralleled by lack of resistance to gentamicin. The decreased gentamicin resistance level in *E. coli* transformants may be due to the lack of enzyme synthesis.

Not only plasmids described in this paper but also other *P. aeruginosa* plasmids mediating gentamicin resistance which can replicate in *E. coli* have been reported to confer a lower level of gentamicin resistance in *E. coli* than *P. aeruginosa* (19, 26). When the level of gentamicin resistance is expressed as the ratio of MIC values of an R^+ host to that of an R^- host to compare the relative level of resistance, the relative value for *P. aeruginosa* strains carrying pMS106, pMS112, pMG1 (18), pMG2 (18), RPL11 (13), R151 (13), Rms149 (13), or R1033 (26) is 16, 64, 40, 40, 32, 32, 16, and 330, respectively, and that for *E. coli* carrying the same plasmids is 1, 4, >8, >8, 8, 4, 2, and 125, respectively. The resistance level of these plasmids, except for R1033, is similar to that of the plasmids described in this paper. Although the precise mechanisms of aminoglycoside resistance caused by enzymatic modification are not completely known, the rate of aminoglycoside uptake relative to the rate of drug modification seems to be an important variable (7). A difference in the rate of this uptake could be one of the reasons for the observed host dependency of the gentamicin resistance level. In addition, a decreased resistance could be due to other factors, such as reduced or absent modifying enzyme synthesis.

That phenotypic properties of plasmids or chromosomal genes change in different species of bacteria has been described previously. Hedges et al. (11) pointed out an impediment in the expression of *P. aeruginosa* tryptophan synthase genes in *E. coli*. A low level of gentamicin resistance in *Vibrio cholerae* compared with *E. coli* (10) and a lack of expression of *E. coli* genes in *B. subtilis* have also been reported (8). In these cases and in the results described in this paper, the mechanisms have not been elucidated, but some defect at the transcriptional level in different hosts is suggested.

ACKNOWLEDGMENT

This work was supported in part by Grant-in-Aid for Scientific Research 56030023 from the Ministry of Education, Science and Culture, Japan.

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