ISOLATION AND CHARACTERIZATION OF A STREPTOMYCIN RESISTANCE PLASMID FROM *PSEUDOMONAS CEPACIA*

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A plasmid, Rms425, mediating resistance to streptomycin(Sm) and mercury(Hg) was isolated from *Pseudomonas cepacia* GN11131 of clinical origin. Rms425 was transferred to *Pseudomonas cepacia, Pseudomonas aeruginosa* and *Escherichia coli* strains by transformation with purified DNA and by conjugation between isogenic strains of *P. aeruginosa* or of *E. coli* by mixing on membrane filters. The molecular weight of Rms425 was estimated to be about 32 megadaltons by electron microscopy and it was classified as incompatibility group P.

Examining the incorporation of [γ-^{32}P] or [8-^{14}C] from isotope-labelled ATP into Sm using the cell-free extracts from *P. cepacia* or *E. coli* strains harboring Rms425, we found that the Sm resistance conferred by Rms425 was due to the phosphorylation of the drug.

Pseudomonas cepacia strains have recently been isolated with increasing frequency from clinical specimens, and most of them are found to be resistant to antimicrobial agents, especially to aminoglycoside antibiotics.

We wished to know whether the resistance to aminoglycosides was mediated by plasmids, and found that among five representative strains of clinical origin resistant to Sm, Km(kanamycin) or Gm (gentamicin) one strain carried a plasmid mediating Sm(streptomycin) resistance. GONZALEZ *et al.*[1] isolated plasmids from several strains of *P. cepacia,* but no phenotypic function was assigned to any of these plasmids. The first isolation of drug-resistant plasmids from tetracycline or ampicillin resistant *P. cepacia* strains was reported by WILLIAMS *et al.*[2]. This paper describes the properties of the plasmid mediating Sm resistance in *P. cepacia* GN11131 and the biochemical mechanism of the Sm resistance mediated by this plasmid.

Materials and Methods

Bacterial Strains

Pseudomonas cepacia strains were isolated from clinical specimens and stocked in this laboratory. *Pseudomonas aeruginosa* PAO2142 (*met, ilv, lys*) strains resistant to rifampicin (Rp^r) or to nalidixic acid (Na^r), *E. coli* K-12 strains χ1037 (*gal, hsd, lac, met, rel, sup*) and ML1410 (Na^r, *met*), and *P. cepacia* ML 5062 Rp^r were used for transformation and mating experiments. ML5062 is a mutant of GN11131 which spontaneously lost resistance to both Sm and Hg. R Factors belonging to different incompatibility groups were used to determine the incompatibility type of the R plasmids. These were kindly supplied by N. DATTA (Royal Postgraduate Medical School, London). Incompatibility tests were carried out by the method of DATTA and HEDGES[3,4].

Chemicals

Streptomycin (Sm), spectinomycin (Sp), kanamycin (Km), tetracycline (Tc), chloramphenicol (Cm), sulfonamide (Su), gentamicin (Gm), carbenicillin (Cb), ampicillin (Ap), nalidixic acid (Na), and rifampicin (Rp) were used. Mercuric chloride (Hg) was obtained commercially. The isotope-labelled

preparations of ATP, *i.e.*, [γ-^{32}P] ATP (926 μCi/ml) and [8-^{14}C] ATP (50 μCi/ml), were purchased from the Radiochemical Centre, Amersham, England.

Media

Antibiotic medium 3 (Difco) and L-broth were used for liquid cultures of bacterial strains. For the determination of drug resistance, heart infusion agar (Eiken Chemical Co., Tokyo) and peptone water were used. The peptone water consisted of 1,000 ml of distilled water, 5 g NaCl, and 10 g peptone. Bromothymol blue (BTB)-lactose agar containing selective drugs was used as the selective medium for the transformants or transconjugants. BTB agar consisted of nutrient broth (10 g beef extract, 10 g peptone and 3 g NaCl in 1,000 ml distilled water) supplemented with 80 mg BTB and 15 g agar.

Determination of Drug Resistance

Drug resistance was determined by the agar dilution method, and the level of resistance was expressed as the minimum inhibitory concentration (MIC) of a drug. An overnight culture in peptone water was diluted 10,000-fold with BSG, which consisted of 1,000 ml of distilled water, 8.5 g NaCl, 0.3 g KH_2PO_4, 0.6 g Na_2HPO_4, and 0.1 g gelatin. One loopful (5 μl) of the diluted culture was spotted on a series of agar plates containing serial two-fold dilutions of a drug. After 18 hours of incubation at 37°C, the bacterial growth was scored.

Isolation of Plasmid DNA

Plasmid DNA was isolated from *P. cepacia* GN11131 and *P. aeruginosa* PAO2142 carrying the R plasmid. Cells were lysed with a Triton X-100, and after sedimenting the bulk of chromosomal DNA, plasmid DNA was separated and purified by density gradient centrifugation in cesium chloride in the presence of ethidium bromide, as described by KUPERSZTOCH *et al.*[5].

Transformation

Transformation of (Sm·Hg) resistance in *P. cepacia* or *P. aeruginosa* with plasmid DNA was followed the method described by SANO and KAGEYAMA[6], and that of *E. coli* K-12 χ 1037 was followed the method described by COHEN *et al.*[7]. BTB lactose agar supplemented with Rp (100 μg/ml) and either Sm (25 μg/ml) or Hg (12.5 μg/ml) was used for the selective plates.

Transfer of Drug Resistance

The transfer in liquid medium was carried out by mixing one part of the donor culture and nine parts of the recipient culture in the stationary phase of growth, followed by 2 hours incubation at 30°C or 37°C. Transfer on the membrane-filter was performed as follows. Five-milliliter portions of the 1: 9 mixtures of donor and recipient cultures in the stationary phase were filtered through Millipore filters (Millipore Corp., pore size: 0.45 μm). The membranes which retained bacterial cells were incubated at 30°C or 37°C for 2 hours on the surface of BTB-lactose agar plates. Appropriate dilutions of the suspension were plated on BTB-lactose agar containing drugs. Concentration of drugs (μg/ml) for the selection of transconjugants were Na (100 μg/ml), Sm (12.5 μg/ml), and Hg (12.5 μg/ml) for *E. coli* ML1410 (Na^r), and Sm (12.5 μg/ml), Hg (12.5 μg/ml), and Na (400 μg/ml) for *P. aeruginosa* PAO2142 Na^r.

Electron Microscopy

Contour lengths were measured by enlarging the photographs of circular DNA molecules. The double-stranded replicative form of a phage fd DNA[8] was used as standard for the length measurement of the molecules.

Preparation of the S-30 Fraction and Inactivation Reaction of Sm

The S-30 fractions, the supernatant of 30,000$\times g$ centrifugation, from *P. cepacia, P. aeruginosa,* and *E. coli* strains were prepared by the procedure described previously[9,10]. The reaction mixture consisted of 0.2 ml of 0.5 M tris-HCl buffer (pH 8.0), 0.05 ml of 20 mM ATP, 0.05 ml of 0.02 M magnesium acetate, 0.05 ml of 0.5 mM Sm, and 0.15 ml of the S-30 fraction. After incubation at 37°C for 5 hours, the reaction was stopped by heating in boiling water for 1 minute. Residual antibiotic activity in the reaction mixture was determined by bioassay using *Bacillus subtilis* ATCC6633.

Radio-isotope Assay for ATP Incorporation into Sm

The incorporation of [^{14}C]ATP or [γ-^{32}P] ATP into Sm by Sm-inactivating enzyme was carried

out by the procedure described previously[11]. The reaction mixture consisted of 30 μl of the S-30 fraction (10 mg of protein/ml), 10 μl of 0.02 M magnesium acetate, 10 μl of 0.5 mM Sm, 40 μl of 0.5 M tris-HCl buffer (pH 8.0), and 1 μl of [γ-^{32}P] ATP or 10 μl of [^{14}C] ATP. The reaction was carried out at 37°C for 1 hour. The reaction mixture was pipetted onto a disk of phosphocellulose paper (Whatman P-81), washed with distilled water, and dried. Radioactivity was counted in a toluene-based scintillator with a liquid scintillation counter (Packard Instrument).

Results

Inactivation of Sm by *P. cepacia* Strains

We selected five *P. cepacia* strains from our stock cultures (80 strains) with reference to patterns of resistance to aminoglycoside antibiotics such as Sm, Km, and Gm (Table 1). Among the strains tested, *P. cepacia* GN11131 was Sm resistant but sensitive to Km and Gm and inactivated Sm; the remaining four strains did not inactivate Sm. No strains were found that would inactivate either Km or Gm. It was found that the Sm resistance of *P. cepacia* GN11131 was lost simultaneously with Hg resistance after storage in a cooked meat medium (Eiken) at 20°C for several months.

Table 1. Patterns of resistance to aminoglycoside antibiotics in *P. cepacia* and inactivation of Sm.

Bacterial strains	MIC (μg/ml)			Inactivation* of		
	Sm	Km	Gm	Sm	Km	Gm
GN11121	400	6.25	100	—	—	—
GN11129	200	3.13	50	—	—	—
GN11131	400	1.56	1.56	+	—	—
GN11140	800	50	200	—	—	—
GN11171	800	200	200	—	—	—

* See Materials and Methods for details.

Isolation of Plasmid DNA

P. cepacia GN11131 cells possessing (Sm·Hg) resistance were lysed with Triton X-100 and the DNA was concentrated with polyethylene glycol. Two bands could be seen in the cesium chloride-ethidium bromide equilibrium density gradients. Each of the bands was collected, examined under electron microscopy, and used for transformation. We found the circular DNA as an OC form in the upper band, and only sheared fragments were detected in the lower band.

Using the 20 μl of upper-band DNA, (Sm·Hg) resistance could be transferred to *P. aeruginosa* PAO2142 Rp^r and *P. cepacia* ML5062 Rp^r by transformation (Table 2, Exp. 1). CCC plasmid DNA could be detected in cesium chloride density gradients of the DNA form PAO2142 Rp^r transformants. This plasmid was called Rms425.

Table 2. Transformation of *Pseudomonas* and *E. coli* strains with DNAs from (Sm·Hg) resistant strains.

Exp. No.	DNA from	Recipient	Transformants obtained		
			Number	Resistance** pattern	Sm inactivation
1	*P. cepacia* GN11131	*P. cepacia* ML5062 Rp^r	2	Sm, Hg	+
		P. aeruginosa PAO2142 Rp^r	10	Sm, Hg	+
2	*P. aeruginosa** PAO2142 Rp^r (Sm·Hg)^r	*P. cepacia* ML5062 Rp^r	50	Sm, Hg	+
		P. aeruginosa PAO2142 Rp^r	220	Sm, Hg	+
		E. coli K-12 χ1037 Rp^r	50	Sm, Hg	+

* A transformant obtained by Exp. 1. The ccc DNA from PAO2142 Rp^r (Sm·Hg)^r was used for transformation to each recipient culture.

** All colonies of the transformants were examined.

Resistance Conferred by Rms425

The plasmid DNAs from PAO2142 Rp^r $(Sm \cdot Hg)^r$ were used for further transformation of the recipient strains of *P. cepacia, P. aeruginosa,* and *E. coli* (Table 2, Exp. 2). The number of transformants of *P. cepacia* ML5062, *P. aeruginosa* PAO2142 Rp^r, and *E. coli* χ1037 Rp^r were 10, 220, and 50 colonies per recipient culture (10^7 cells), respectively. All were resistant to both Sm and Hg, even when selected with either drug, indicating that the genes governing resistance to Sm and Hg were located on plasmid Rms425. The transformants were sensitive to all of the other antibiotics tested, *i.e.*, Gm, Km, Sp, Su, Tc, Cb, and Ap. All transformants of *P. cepacia, P. aeruginosa,* and *E. coli* inactivated Sm.

Genetic Properties of Rms425

The transferability of (Sm·Hg) resistance in *P. cepacia* GN11131 and in the transformants of *P. aeruginosa* PAO2142 Rp^r and *E. coli* χ1037 Rp^r was examined using *P. cepacia* ML 5062 Rp^r, *P. aeruginosa* PAO2142 Na^r, and *E. coli* ML 1410 as recipient strains by mixing in liquid medium or on membrane filters. The results obtained by mixing on membrane filters are shown in Table 3. No transfer from *P. cepacia* GN11131 to various recipients was detected. However, *P. aeruginosa* PAO2142 Rp^r $Rms425^+$ could transfer its resistance to *P. aeruginosa* PAO2142 Na^r at a low frequency. Similarly, *E. coli* χ1037 Rp^r $Rms425^+$ could transfer the plasmid to *E. coli* ML1410 Na^r. But Rms425 was non-

Table 3. Transfer of (Sm·Hg) resistance by mixing on membrane filters.

Donor	Recipient	Transfer frequency*	Resistance pattern
P. cepacia GN11131	*P. cepacia* ML5062 Rp^r	$<10^{-8}$	—
	P. aeruginosa PAO2142 Rp^r	$<10^{-8}$	—
	E. coli K12 χ1037 Rp^r	$<10^{-8}$	—
	E. coli K12 ML1410	$<10^{-8}$	—
P. aeruginosa PAO2142 Rp^r $Rms425^+$	*P. aeruginosa* PAO2142 Na^r	1×10^{-7}	Sm, Hg
	E. coli K12 ML1410	$<10^{-8}$	—
E. coli K12 χ1037 Rp^r $Rms425^+$	*P. aeruginosa* PAO2142 Na^r	$<10^{-8}$	—
	E. coli K12 ML1410	2×10^{-7}	Sm, Hg

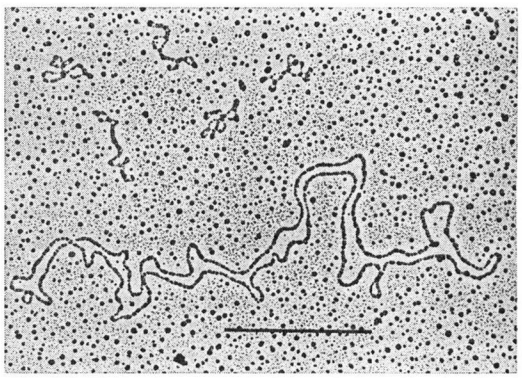
* Transfer frequency was expressed as the ratio of the number of transconjugants to that of donor cells.

transmissible between *P. aeruginosa* and *E. coli* strains. When mixed in liquid medium, no transfer was detectable in all combinations of donors and recipients when selected by either Sm or Hg.

Incompatibility testing of Rms425 was performed using indicator R plasmids belonging to different incompatibility groups. Each of these R plasmids was conjugally transferred to *E. coli* χ1037 Rp^r or *E. coli* χ1037 Rp^r $Rms425^+$.

Only the transfer frequency of RP4 (group P) was reduced (approximately 1,000-fold by the presence of Rms425 in the recipients, and (Sm·Hg) resistance conferred by Rms425 was

Fig. 1. Electron micrograph of a circular DNA molecule of Rms425 plasmid isolated from *P. aeruginosa* PAO2142 $Rms425^+$.
Bar represents 1 μm.



eliminated from the transconjugants by the incoming RP4. On the contrary, the transfer frequencies of R40a (group C), N-3 (group N), S-a (group W), R391 (group J), or R1 (group FII) were not reduced by Rms425, and each of these plasmids coexisted with Rms425 in the recipients. We therefore conclude that Rms425 belonged to the incompatibility group P.

The molecular nature of Rms425 was examined by electron microscopy and its open circular form is shown in Fig. 1. The average molecular weight was estimated as 32.4×10^6 daltons when 18 molecules of DNA were measured.

Inactivation Mechanisms of Sm by Rms425

We investigated the biochemical mechanisms of Sm resistance by incubation with cell-free extracts from *P. aeruginosa* PAO2142 Rms425$^+$ and *E. coli* χ1037 Rms425$^+$.

The results indicated that Rms425 encoded an enzyme that inactivated Sm by phosphorylation (Table 4).

Table 4. Incorporation of [γ-^{32}P]ATP into Sm by cell-free extract from strains carrying Rms425.

Strains	Incorporation of radioisotope [^{14}C] or [^{32}P] (cpm) of	
	[^{14}C]ATP	[γ-^{32}P] ATP
P. aeruginosa PAO2142 Rms425$^+$	129	24,803
P. aeruginosa PAO2142	98	58
E. coli χ1037 Rms425$^+$	121	19,987
E. coli χ1037	87	62

Discussion

Plasmids have been reported in *P. cepacia*[1]; however, the isolation of drug-resistance plasmids from *P. cepacia* strains has only been described by WILLIAMS *et al.*[12]. The plasmids, pJW2 and pJW3, mediated, respectively, resistance to tetracycline and ampicillin.

We have isolated a resistance plasmid Rms425 which mediated (Sm·Hg) resistance from a clinical isolate of *P. cepacia* GN11131. The plasmid DNA was clearly detected in *P. aeruginosa* strains after transformation with GN11131 DNA. Difficulties in the isolation of the plasmid DNA as a ccc form from *P. cepacia* strains might be due to nuclease activity of *P. cepacia* as described by WILLIAMS *et al.*[12]. The molecular weight of Rms425 isolated from *P. aeruginosa* PAO2142 transformants was 32.4 Mdal; larger than pJW2 and pJW3, which were presumed to be nonconjugative because of their smaller sizes, *i.e.*, 2.9 Mdal and 5.4 Mdal, respectively.

Plasmid Rms425 belongs to incompatibility group P, and was classified in the P-1 group in *P. aeruginosa*. As reported by DATTA *et al.*[13,14], the R plasmids of incompatibility group P could be transferred to *P. aeruginosa* as well as *E. coli*. Rms425 might be slightly defective in transferability, because it was only transmissible between isogenic strains, *i.e.*, between *P. aeruginosa* or between *E. coli* at an extremely low frequency by filter mating, but not between heterogenic species or by mixing in broth. Furthermore, Rms425 was not transmissible from *P. cepacia* GN11131 to any of the organisms tested, including an isogenic strain ML5062 Rp^r. On the other hand, Rms425 was able to replicate and express (Sm·Hg) resistance in *P. cepacia*, *P. aeruginosa*, and *E. coli* and the transformants carrying Rms425 stably maintained their (Sm·Hg) resistance in the new hosts.

Enzymatic inactivation of aminoglycoside antibiotics is considered to be one of the main mechanisms of resistance in Gram-negative and Gram-positive pathogens isolated from clinical specimens. However, we could not detect any enzymes inactivating Sm, Km or Gm in resistant *P. cepacia* strains with the exception of GN11131. SM-inactivating enzymes in Sm-resistant strains of *E. coli*[15], *P. aeruginosa*[16,17], *P. lachrymans*[18], and *Staphylococcus aureus*[11], have two mechanisms, either pho-

sphorylation or adenylylation, *i.e.*, due to Sm3″-phosphotransferase, Sm6-phosphotransferase, Sm3″-adenylyltransferase, or Sm6-adenylyltransferase. Since enzymatic transfer of the [γ-^{32}P] but not [8-^{14}C] from isotope-labelled ATP into Sm was accomplished with extracts of Rms425 containing strains, we concluded that Rms425 encoded an Sm phosphotransferase.

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References

1) GONZALEZ, C. F. & A. K. VIDAVER: Bacteriocin, plasmid and pectolytic diversity in *Pseudomonas cepacia* of clinical and plant origin. J. Gen. Microbiol. 110: 161~170, 1979
2) WILLIAMS, J. A.; J. P. YEGGY, C. C. FIELD & A. J. MARKOVETZ: Resistance plasmids in *Pseudomonas cepacia* 4G9. J. Bacteriol. 140: 1017~1022, 1979
3) DATTA, N. & R. W. HEDGES: Compatibility groups among *fi*$^-$ R factors. Nature (London) 234: 222~223, 1971
4) DATTA, N. & R. W. HEDGES: Trimethoprim resistance confered by W plasmids in *Enterobacteriaceae*. J. Gen. Microbiol. 72: 349~356, 1972
5) KUPERSZTOCH-PORTONY, Y. M.; M. A. LOVETT & D. R. HELINSKI: Strand and site specificity of the relaxation event for the relaxation complex of the antibiotic resistance plasmid R6K. Biochemistry 13: 5484~5490, 1974
6) SANO, Y. & M. KAGEYAMA: Transformation of *Pseudomonas aeruginosa* by plasmid DNA. J. Gen. Appl. Microbiol. 23: 183~186, 1977
7) COHEN, S. N.; A. C. Y. CHANG & L. HSU: Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. 69: 2110~2114, 1972
8) BECK, E.; R. SOMMER, E. A. AUERSWALD, C. KURZ, B. ZINK, G. OSTERBURG, H. SHALLER, K. SUGIMOTO, H. SUGISAKI, T. OKAMOTO & M. TAKANAMI: Nucleotide sequence of bacteriophage fd DNA. Nucleic Acids Res. 5: 4495~4503, 1978
9) KAWABE, H. & S. MITSUHASHI: Acetylation of dideoxy-kanamycin B by *Pseudomonas aeruginosa*. Jpn. J. Microbiol. 16: 436~437, 1972
10) SAGAI, H.; V. KRCMERY, H. HASUDA, S. IYOBE, H. KNOTHE & S. MITSUHASHI: R Factor-mediated resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*. Jpn. J. Microbiol. 19: 427~432, 1975
11) KAWABE, H.; M. INOUE & S. MITSUHASHI: Inactivation of dihydrostreptomycin and spectinomycin by *Staphylococcus aureus*. Antimicrob. Agents Chemother. 5: 553~557, 1974
12) WILLIAMS, J. A.; J. P. YEGGY & A. J. MARKOVETZ: Role of nucleases in the isolation of plasmid deoxyribonucleic acid from *Pseudomonas cepacia* 4G9. J. Bacteriol. 141: 1057~1059, 1980
13) DATTA, N. & R. W. HEDGES: Host ranges of R factors. J. Gen. Microbiol. 70: 453~460, 1972
14) DATTA, N.; R. W. HEDGES, E. J. SHAW, R. B. SYKES & M. H. RICHMOND: Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. 108: 1244~1249, 1971
15) OZANNE, B.; R. BENVENISTE, D. TIPPER & D. R. HELINSKI: Strand and site specificity of the relaxation event for the relaxation complex of the antibiotic resistance plasmid R6K. Biochemistry 13: 5484~5490, 1969
16) KAWABE, H.; F. KOBAYASHI, M. YAMAGUCHI, R. UTAHARA & S. MITSUHASHI: 3″-Phosphoryl-dihydrostreptomycin produced by the inactivating enzyme of *Pseudomonas aeruginosa*. J. Antibiotics 24: 651~652, 1971
17) KIDA, M.; T. ASAKA, M. YONEDA & S. MITSUHASHI: Phosphorylation of dihydrostreptomycin by *Pseudomonas aeruginosa*. Microbial Drug Resistance. p. 441. University of Tokyo Press, Tokyo, 1975
18) KAWABE, H.; H. SAKURAI, K. FUKASAWA, S. SHIMIZU, K. HASUDA, S. IYOBE & S. MITSUHASHI: Phosphorylation and inactivation of streptomycin by plant pathogenic *Pseudomonas lachrymans*. J. Antibiotics 32: 425~426, 1979