MECHANISMS OF STREPTOMYCIN(SM)-RESISTANCE OF HIGHLY SM-RESISTANT *PSEUDOMONAS AERUGINOSA* STRAINS

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Three clinical isolates, K-Ps 94, K-Ps 97 and K-Ps 102, of *Pseudomonas aeruginosa* having R factor and showing MIC of more than 51,200 mcg/ml to streptomycin (SM), were examined for mechanisms of SM-resistance. Among the strains, K-Ps 94 and K-Ps 102 had R factor conferring SM-resistance. In K-Ps 94, the mechanism of SM-resistance was mainly owing to SM-phosphorylating enzyme and also owing to decreased permeability by an R factor, kR94. In K-Ps 97, it was considered to be due to SM-adenylylating enzyme by the chromosomal gene but not R factor, kR97. In K-Ps 102, the reduced permeability of the cell membrane to SM by an R factor, kR102, and the reduced affinity of the ribosome to the drug by the chromosomal gene contributed to the mechanisms of SM-resistance.

The inactivation of streptomycin (SM) by phosphorylation and adenylylation of 3″-OH group of N-methyl-L-glucosamine has been reported as the resistance mechanism for SM in clinical isolates.[1] SM-resistance of *Pseudomonas aeruginosa* has been investigated in detail by TSENG *et al.*[2] These authors found that SM-resistance was mainly due to reduction of the membrane permeability in the strains showing minimum inhibitory concentration (MIC) less than 12.5 mcg/ml, and due to phosphorylation of SM in the strains showing MIC about 500~2,000 mcg/ml by the presence of R factor. In the resistant strains showing MIC of 20,000 mcg/ml R factor did not exist and the resistance mechanism was reported to be due to the change of ribosomes.

The present authors tried to detect R factor from *P. aeruginosa* obtained from clinical specimens, and found R factors resistant to various drugs in about 10 per cent of the strains.[3] Tetracycline (TC)-resistance mechanism of the strain having an R factor, kR102, resistant to five drugs among the above-described strains was reported previously.[4] This report deals with the mechanisms of drug resistance of highly SM-resistant strains showing MIC more than 51,200 mcg/ml.

Materials and Methods

Strains used[3]: Table 1 shows the strains of *P. aeruginosa* used in the experiments, their MIC's against SM and their resistance to antibiotics. *Bacillus subtilis* PCI-219 was used for bioassay of SM.

Media: Brain heart infusion (Difco) containing 0.4% KNO_3 (K-BHI) was used, if not otherwise stated. For the multiplication of phage f2 with *Escherichia coli* K12 W1895 Hfr C (nalidixic acid resistance) and for the preparation of ribosomes, tryptone medium (Polypeptone 10 g, NaCl 8 g, yeast extract powder 1 g, glucose 1 g, 1 M $CaCl_2$ 2 ml; added after sterilization) was used. Peptone water and nutrient agar (Eiken, Tokyo) were used for determination of MIC. For spheroplast formation, medium B (normal medium, Eiken), medium BS (medium B

containing 12 % sucrose) and medium BGS (medium BS containing 3 % glycine) were used. When using as solid media, 1.5 % agar was added to each medium.

Reagents: SM of Meiji Seika Co., Ltd. was used. ATP (adenosine triphosphate), GTP (guanosine triphosphate), transfer RNA (t-RNA) from *E. coli* W, creatine phosphate, and creatine phosphokinase used in the experiments were all products of Sigma Chemicals Co. Phospho-(enol)pyruvate and ^{14}C-L-valine (specific activity, 225 mCi/mmole) were obtained from Daiichi Pure Chemicals Co., Ltd., and ATP-γ-^{32}P (specific activity, 2.65 Ci/mmole) and ATP-8-^{14}C (specific activity, 45 Ci/mmole) were obtained from the Radiochemical Centre, Amersham and from New England Nuclear, respectively.

Drug resistance: MIC was determined according to the method reported previously,[3] and drug resistance was represented by MIC. In some experiments multiplication of bacteria in liquid medium, K-BHI, was compared according to TREFFER's method[5]: the resistance among strains was compared by means of ID_{50}, the dose for 50 % inhibition of growth.

Preparation of cell-free extract: The extract was prepared according to the method of O'HARA *et al.*[6] Protein content was determined by the method of LOWRY *et al.*[7]

Inactivation of SM by cell-free extract: Three-tenth ml of the cell-free extract prepared to contain 30 mg protein/ml, 0.1 ml of 40 mM ATP, 0.1 ml of 1 mM SM, and 0.5 ml of TMK solution (0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride (Tris) buffer, pH 7.8, 0.06 M KCl, 0.01 M magnesium acetate, 6 mM 2-mercaptoethanol) were mixed, and allowed to react at 37°C for 24 hours. After heating at 100°C for 3 minutes, the residual potency of SM was determined with *B. subtilis* PCI-219.[6]

Incorporation of labelled ATP into SM: The reaction mixture consisted of 5 μl of the cell-free extract, 5 μl of labelled ATP (ATP-γ-^{32}P or ATP-8-^{14}C, 1 μCi), 5 μl of 1 mM SM and 35 μl of TMK solution. After the reaction was carried out at 37°C for 1 hour, 10 μl of the mixture was taken out, adsorbed by phosphocellulose paper (Whatman P81) of 0.75 cm^2, rinsed with 20 ml of hot water 5 times, and dried, followed by counting radioactivity by Packard Tri-carb Scintillation Spectrometer (Model 3330). Toluene based fluid was prepared by dissolving 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of toluene. The non-specific binding of labelled ATP was determined by the reaction in the absence of enzyme or SM.[6]

Bioassay of SM in the culture: One-tenth ml of the overnight culture of each strain and 0.1 ml of 500 mcg/ml of SM were added to 0.8 ml of K-BHI, and after incubating at 37°C for 18 hours, the solution was heated at 100°C for 3 minutes, and centrifuged at 50,000$\times g$ for 15 minutes. The residual potency of SM in the supernatant thus obtained was determined by bioassay.

Incorporation of amino acids: Phage f2 was isolated according to the method of GESTLAND,[8] and RNA was extracted from the phage using the method of WEBSTER *et al.*[9] S 100 fraction and ribosomes were prepared according to the method of MODOLELL *et al.*[10] When crushing the bacteria, we added Bentonite (final concentration: 25 mg/ml) prepared by the method of FRAENKEL-CONRAT *et al.*[11] as inhibitor of RNase activity and deoxycholate (final concentration: 0.5 %) for easy separation of ribosomes from the cell membrane. The composition of the reaction mixture is as follows: 60 mM Tris buffer, pH 7.8; 30 mM NH_4Cl; 10 mM Mg-acetate; 10 mM reduced glutathione; 3 mM ATP; 0.2 mM GTP; 10 mM phospho(enol)pyruvate; 5 mM creatine phosphate; 50 mcg creatine phosphokinase/ml; 0.05 mM each of the 19 amino acids; 0.03 mM ^{14}C-valine; 0.1 mg of f2 RNA/ml; 1 mg t-RNA/ml; 0.2 volume of S 100 and ribosomal suspension (10 mg/ml). When addition of SM was required, it was kept at 37°C for 10 minutes during preincubation, and then f2 RNA and ^{14}C-valine were added to initiate the reaction.

The reaction was performed at 34°C for 30 minutes: then 50 μl of the reaction mixture was removed, and after adding 0.5 ml of cold trichloroacetic acid (TCA), the mixture was heated at 90°C for 15 minutes, and filtered through a membrane filter (Toyo Roshi, Co., Ltd.: TM-2, 0.42 μ, size 13 m/m). After washing with 1 ml of 5 % TCA four times and drying, the

filter was put in 10 ml of toluene based fluid and the radioactivity was determined by the liquid scintillation spectrometer.

Formation of spheroplasts: Formation of spheroplasts by glycine treatment was performed according to the method of KAWAKAMI *et al.*[12]

Results

As shown in Table 1, according to the results of the comparison between the clinical isolates and the conjugant strains, it was presumed that K-Ps 94 would have R factor conferring high SM-resistance and KM-resistance, and that in K-Ps 97 the SM-resistance was non-transferable and possibly to be due to the control of the chromosomal gene. MIC of a conjugant strain, K-Ps 47 RFP (kR102), was 1,600 mcg/ml, which was obviously higher than that of K-Ps 47 RFP, 50 mcg/ml, but was lower than that of the original strain, K-Ps 102, more than 51,200 mcg/ml. Therefore, the SM-resistance of K-Ps 102 seemed to be due to the control of the resistance genes of R factor and chromosome.

Table 1. *Pseudomonas aeruginosa* strains used

	Clinical isolates		Conjugants***	
Strain No.	MIC (mcg/ml) of SM	Resistance to*	Strain No.	MIC (mcg/ml) of SM
K-Ps 61	6,400	TC CP SM SA	K-Ps 47 RFP (kR61)	6,400
K-Ps 79	1,600	CP SM SA	K-Ps 47 RFP (kR79)	1,600
K-Ps 94	>51,200	CP SM KM	K-Ps 47 RFP (kR94)	>51,200
K-Ps 97	>51,200	SM KM SA	K-Ps 47 RFP (kR97)	100
K-Ps 102	>51,200	TC CP SM SA KM	K-Ps 47 RFP (kR102)	1,600
K-Ps 47 RFP**	50			

* Abbreviations: TC, tetracycline; CP, chloramphenicol; SA, sulfonamide; KM, kanamycin
Underlines indicate the resistance markers, which appeared in conjugants.

** Rifampicin (RFP)-resistant mutant from clinical isolate, K-Ps 47.

*** Conjugants were obtained from clinical isolates resistant to SM by mating with K-Ps 47 RFP as described previously.[8]

As the mechanism of SM-resistance, inactivation due to SM-phosphorylating or adenylylating enzyme is known; it was investigated with ATP-γ-^{32}P and ATP-8-^{14}C in this experiment. The results are shown in Table 2. As the degree of phosphorylation of SM was similar in K-Ps 94 and K-Ps 47 RFP (kR94), it was found that the mechanism of SM-resistance in K-Ps 94 was mainly owing to R factor controlling the production of SM-phosphorylating enzyme. It was also confirmed that in K-Ps 97, the production of adenylylating enzyme by SM-resistance gene on the chromosome would be the main mechanism of SM-resistance. It was, however, suggested that in K-Ps 102 SM-resistance might be due to some mechanism(s) other than SM-inactivating enzyme.

It has been reported that spheroplasts of *E. coli* having R70 factor show a great increase of susceptibility to chloramphenicol (CP) as compared with intact cells of the same bacteria.[13]

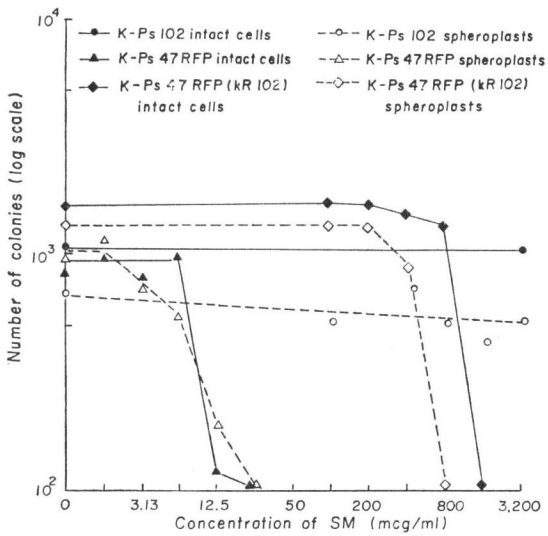
Table 2. Incorporation of labelled ATP into SM and inactivation of the drug by cell-free extracts from SM-resistant strains of *P. aeruginosa*

Cell-free extract from	Incorporation of*		Inactivation**
	ATP-γ-^{32}P	ATP-8-^{14}C	
K-Ps 61	835	126	+
K-Ps 79	0	25	−
K-Ps 94	2,856	107	+
K-Ps 97	0	1,848	+
K-Ps 102	30	5	−
K-Ps 47 RFP	0	60	−
K-Ps 47 RFP (kR94)	3,067	133	+
K-Ps 47 RFP (kR102)	139	25	−

* Counts were presented by counts per minute of ^{32}P- or ^{14}C-labelled ATP onto phosphocellulose paper. A background incorporation of radioactivity from a control, described in Materials and Methods, was subtracted to obtain the values listed above.

** Determined by a microbioassay method.

Fig. 1. SM resistance of intact cells and glycine-spheroplasts



Susceptibility of spheroplasts, obtained by glycine treatment, was investigated to clarify the mechanism of resistance to SM as to K-Ps 102. The results were shown in Fig. 1. K-Ps 102 did not show any susceptibility to SM in either intact cells or spheroplasts at a concentration lower than 3,200 mcg of SM/ml. Similarly in K-Ps 47 RFP the susceptibility was not different between the intact cells and the spheroplasts. As to K-Ps 47 RFP (kR102), the susceptibility tends to increase slightly in its spheroplasts, but the difference was not so great between the intact cells and spheroplasts.

The drug susceptibility of *E. coli* is reported to increase by EDTA treatment.[14] In the experiment reported here, the concentration of EDTA was determined to be 0.5 mM, which allowed 30% growth of *P. aeruginosa*, and the susceptibilities to SM of various strains were compared. The SM susceptibility was represented by ID_{50} in Table 3. The resistance due to R factor in K-Ps 102 could be evaluated based on the SM-resistance of K-Ps 47 RFP (kR102), as described above. In the conjugant, the susceptibility was obviously increased by EDTA treatment up to the level (23 mcg/ml) almost similar to ID_{50} of K-Ps 47 RFP, 12.5 mcg/ml. In the R factor-having strain, K-Ps 102, the susceptibility was obviously increased by EDTA treatment from ID_{50} of 19,000 to 800 mcg/ml. The difference of ID_{50} between intact cells and spheroplasts in both strains was due to the presence of R factor, kR 102, and the mechanism of SM-resistance of the R factor was revealed to be due to reduced permeability

Table 3. Comparison of ID_{50} values of SM to strains by treatment with or without EDTA

Condition	ID_{50} (mcg/ml of SM)				
	K-Ps 102	K-Ps 47 RFP	K-Ps 47 RFP (kR102)	K-Ps 94	K-Ps 97
SM only	19,500	25	230	21,000	16,000
SM, EDTA*	800	12.5	23	12,000	8,000

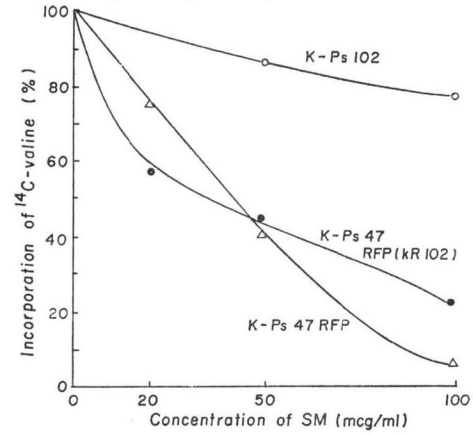
* 0.5 mM EDTA (ethylenediaminetetraacetic acid).

of the cytoplasmic membrane. However, it is reasonable to presume that the resistance of K-Ps 102 after EDTA treatment (ID_{50}: 800 mcg/ml) is substantially due to the resistance mechanism by chromosomal gene. In K-Ps 94 and K-Ps 97 each ID_{50} was reduced to about 1/2, and the effect of EDTA treatment could not be neglected. This will be discussed later.

The change of ribosome will be first considered as the resistance mechanism of chromosome gene in K-Ps 102. The SM-susceptibility of ribosome in the strain was then investigated. The results are shown in Fig. 2. As can be seen in the figure, the protein synthesis, in K-Ps 102, was still at the level of 80 % even in the presence of 100 mcg/ml of SM, and it proved the ribosome resistance to SM.

Fig. 2. Inhibition of amino acid incorporation by streptomycin

The rate of protein synthesis is expressed as percentage of ^{14}C-valine incorporation (cpm) with SM/^{14}C-valine incorporation (cpm) without SM. In case of cell-free systems from K-Ps 102, K-Ps 47 RFP and K-Ps 47 RFP (kR102) strains, the incorporation without SM were 1134, 1317 and 1015 cpm, respectively.



Discussion

TSENG *et al.* (1972)[2] studied SM-resistance in clinical isolates, and reported the strain which showed MIC of 20,000 mcg/ml but had no R factor. The three highly resistant strains, which were found in our laboratory, showed MIC of more than 51,200 mcg of SM/ml: among them K-Ps 102 and K-Ps 94 had R factor conferring SM-resistance. These three highly resistant strains showed different genetic and biochemical resistance mechanisms: in K-Ps 94 the mechanism of SM-resistance was mainly due to the inactivating enzyme controlled by R factor (SM-phosphorylating enzyme), and in K-Ps 97 it was the SM-adenylylating enzyme by the resistance gene of chromosome. In K-Ps 102 both the change of permeability (reduced permeability of the membrane) by the gene of R factor and the change of ribosome (reduced affinity to the drug) by chromosomal gene contribute to the mechanism of the high SM-resistance. When R factor, kR94, found in K-Ps 94 (MIC: more than 51,200 mcg/ml) was transferred to K-Ps 47 RFP, it gave the resistance to SM of the same level and the degree of its inactivation was also similar. However, the SM-phosphorylase activity of K-Ps 94 was merely about three times higher than that of K-Ps 61 (MIC: 6,400 mcg/ml) as shown in Tables 1 and 2, and its susceptibility was reduced to about a half by EDTA treatment.

From the results, it is difficult to consider a certain relation between the MIC and the inactivating activity in K-Ps 94. Therefore, it was considered that R factor (kR94) would also

affect the membrane permeability as well as the production of SM-phosphorylating enzyme. It was also considered that R factor (kR102) of K-Ps 102 did not affect production of inactivating enzyme but controlled decrease of the membrane permeability to give SM-resistance. Considering the fact, the presence of impermeability gene (imp) may be supposed on R factor. The resistance of K-Ps 79 (MIC: 1,600 mcg/ml) shown in Tables 1 and 2 was controlled by R factor (kR79), but inactivation could not be recognized: the level of resistance was similar to that of K-Ps 47 RFP (kR102). Consequently, kR79 may be considered to be similar to kR102. As to SM-resistance mechanism of bacteria having R factor (kR102), the change being susceptible to the drug by EDTA treatment was more remarkable than that by glycine treatment. Therefore, it was considered that cytoplasmic membrane would be more plausible resistance barrier than cell wall or outer layer of cell membrane.

As to the resistance mechanism other than drug-inactivation in *P. aeruginosa*, TANAKA[15] also reported the ribosome resistance to gentamicin. K-Ps 102 used here was also ribosome resistant. The resistance of the strain may be considered to be further increased by R factor.

It will be noticed that some resistant strains, particularly of *P. aeruginosa*, are controlled genetically by R factor and chromosome, and it will present some problems in the further study on the resistance mechanism.

Though the development of novel drugs resistant to antibiotic inactivating enzyme is regarded as of major importance against drug resistant bacteria, a countermeasure should now be considered to overcome antibiotic resistant bacteria, the resistance mechanism of which is closely related to decreased permeability of cell membrane or low affinity of ribosome against antibiotics.

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References

1) BENVENISTE, R. & J. DAVIES: Mechanisms of antibiotic resistance in bacteria. Ann. Rev. Biochem. 1973: 471~506, 1973
2) TSENG, J. T.; L. E. BRYAN & H. M. VAN DEN ELZEN: Mechanisms and spectrum of streptomycin resistance in a natural population of *Pseudomonas aeruginosa*. Antimicr. Agents & Chemoth. 2: 136~141, 1972
3) KONO, M. & K. O'HARA: Prevalence of R factors in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 91: 191~194, 1975
4) O'HARA, K. & M. KONO: Mechanism of tetracycline resistance in *Pseudomonas aeruginosa* carrying an R factor. J. Antibiotics 28: 607~608, 1975
5) TREFFERS, H. P.: The linear representation of dosage-response curves in microbial-antibiotic assays. J. Bact. 72: 108~114, 1956
6) O'HARA, K.; M. KONO & S. MITSUHASHI: Enzymatic inactivation of a new aminoglycoside antibiotic, sisomicin, by resistant strains of *Pseudomonas aeruginosa*. Antimicr. Agents & Chemoth. 5: 558~561, 1974
7) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
8) GESTLAND, R. F.: Some physical properties of bacteriophage R17 and its RNA. J. Mol. Biol. 8: 496~507, 1964
9) WEBSTER, R. E.; D. L. ENGELFARDT & N. D. ZINDER: Amber mutants and chain termination *in vitro*. J. Mol. Biol. 29: 27~43, 1967
10) MODOLELL, J. & B. D. DAVIS: Rapid inhibition of poly peptide chain extension by streptomycin. Proc. Nat. Acad. Sci. 61: 1279~1286, 1968
11) FRAENKEL-CONRAT, H.; B. SINGER & A. TSUGITA: Purification of viral RNA by means of bentonite. Virology 14: 54~58, 1961
12) KAWAKAMI, M.; N. OSAWA & S. MITSUHASHI: Transmission of the drug resistance factor between spheroplasts. Jap. J. Exp. Med. 31: 259~266, 1961

13) NAGAI, Y. & S. MITSUHASHI: New type of R factors incapable of inactivating chloramphenicol. J. Bact. 109: 1~7, 1972
14) LEIVE, L.: Actinomycin sensitivity in *Escherichia coli* produced by EDTA. Biochem. Biophys. Res. Commun. 18: 13~17, 1965
15) TANAKA, N.: Biochemical studies on gentamicin resistance. J. Antibiotics 23: 469~471, 1970