KANAMYCIN-RESISTANCE MECHANISM OF *PSEUDOMONAS AERUGINOSA* GOVERNED BY AN R-PLASMID INDEPENDENTLY OF INACTIVATING ENZYMES

Sir:

We have isolated a strain K-Ps 102 with R-plasmid resistant to the 5 antibiotics, tetracycline (TC), chloramphenicol (CP), streptomycin (SM), sulfonamide (SA) and kanamycin (KM), from clinically isolated *Pseudomonas aeruginosa* strains.[1] The resistance mechanisms of this R-plasmid kR102 have been studied with TC, SM, and CP[2~4].

The results suggest that a decrease of cell permeability for the respective drugs contributes to the resistance. This report deals with the mechanism of KM-resistance by the R-plasmid, kR102, in *Pseudomonas aeruginosa*. A rifampicin (RFP) resistant mutant, K-Ps 47 RFP sensitive to the 5 antibiotics described above was used as an R⁻ strain. A conjugant, K-Ps 47 RFP (kR102), was obtained from K-Ps 47 RFP by mating with K-Ps 102.

KM-inactivation by cell free extracts was examined as follows: 0.3 ml of cell free extract (30 mg protein/ml), 0.1 ml of 40mM ATP, 0.1 ml of 2 mM CoA, 0.1 ml of 1 mM KM, and 0.4 ml of TMK solution (0.06 M KCl, 0.01 M magnesium acetate, and 6 mM 2-mercaptoethanol dissolved into pH 7.8 Tris-HCl buffer solution) were mixed and allowed to react at 37°C for 24 hours, followed by heating to 100°C for 3 minutes. The remaining KM was determined by microbioassay method using *Bacillus subtilis* PCI-219. KM inactivation was not observed with any of the 3 strains.

Phosphorylation and adenylylation were studied by using a reaction mixture comprising 5 μl of cell-free extract, 5 μl of ATP-γ-^{32}P (Radiochemical Centre, Amersham) or ATP-8-^{14}C (New England Nuclear), 5 μl of 1 mM KM and 35 μl of TMK solution with magnesium acetate substituted by $MgCl_2$, and acetylation was examined by using a reaction mixture comprising 5 μl of cell free extract, 5 μl of 40 mM ATP, 5 μl of 1-^{14}C-acetate (Daiichi Kagaku Co.), 5 μl of 2 mM CoA and 30 μl of TMK solution with magnesium acetate substituted by $MgCl_2$. Each reaction mixture was adsorbed on phosphocellulose paper (Whatman P81, 0.75 cm^2), and radioactivity was determined with a Packard Tricarb Scintillation Spectrometer (Model 3330). The results indicated the absence of phosphorylating, adenylylating and acetylating enzymes as shown in Table 1. The incorporation experiments of labelled compounds into KM using *Pseudomonas aeruginosa* TI-13[5] (phosphorylation), *Klebsiella pneumoniae* 3020[6] (adenylylation) and *Pseudomonas aeruginosa* GN269[7] (acetylation) as reference strains were carried out, and the values of incorporation were determined as 2375 cpm, 1580 cpm (unpublished data) and 2288 cpm[7], respectively.

Table 2. Inhibition of amino acid incorporation by KM in cell-free system

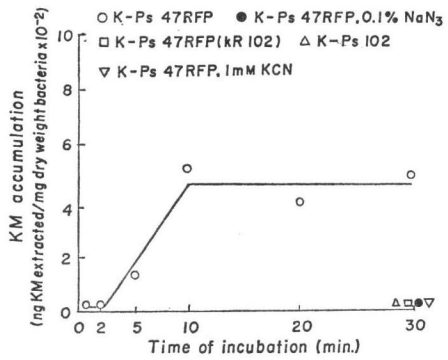
Strain	Concentration of KM(mcg/ml)	Incorporation ^{14}C-valine (cpm)	Inhibition (%)
K-Ps102	0	1295	0
	2	932	28
	20	34	97
	100	12	99
K-Ps 47 RFP	0	1120	0
	2	963	14
	20	12	99
	100	9	>99
K-Ps 47 RFP (kR102)	0	1119	0
	2	940	16
	20	14	99
	100	5	>99

Inhibition of ^{14}C-L-valine (Daiichi Kagaku Co.) incorporation by KM was then examined by the

Table 1. Inactivation of KM of *P. aeruginosa* used.

Strain	Resistance markers (R plasmid)	MIC of KM (mcg/ml)	Inactivation of KM	Incorporation (cpm) of		
				ATP-γ-^{32}P	ATP-8-^{14}C	CH_3COONa-1-^{14}C
K-Ps 102	TC. CP. SM. SA. KM.	800	—	138	30	147
K-Ps 47 RFP		50	—	15	5	127
K-Ps 47 RFP (kR102)	TC. CP. SM. SA. KM.	800	—	0	180	0

Fig. 1. KM-accumulation in KM-broth(500 mcg/ml) with or without NaN_3 or KCN.



method described in the previous paper.[4] Table 2 shows the inhibition of ^{14}C-valine incorporation in the presence of KM, using ribosomes from the three strains. As shown, all 3 strains were found to be sensitive to KM to about the same extent, with 20 mcg/ml of KM inhibiting the protein synthesis almost completely.

Determination of KM incorporation into cells was then examined as follows: KM was added to 250 ml of bacterial culture (nutrient broth) in the exponential phase of growth, at the rate of 500 mcg/ml and 50 ml samples were taken at intervals. The bacteria was collected by centrifugation at $10,000 \times g$ for 10 minutes, and washed 3 times by equal amounts of water. They were then suspended in 1 ml of distilled water and heated to 100°C for 3 minutes to elute KM. The amount of the eluted KM was determined by microbioassay. The amount of KM adsorbed by the cells at zero time was determined and the incorporated KM calculated by subtracting the zero time KM from the total KM eluted. The incorporated KM is expressed as weight per unit of dry weight of bacteria. The results are shown in Fig. 1. With K-Ps 47 RFP, the amount of KM incorporated into cells reached a maximum in 10 minutes, but with K-Ps 102 and K-Ps 47 RFP (kR102), KM was not incorporated at all under the conditions employed. The amounts of KM adsorbed by cells of each strain were 0.3 mcg KM/mg dry weight with K-Ps 47 RFP and K-Ps 47 RFP (kR102), and 0.8 mcg KM/mg dry weight with K-Ps 102. The effect of NaN_3 or KCN, an inhibitor of respiratory enzyme system, on the incorporation of KM into cells was also examined. It was found that the KM incorporation into K-Ps 47 RFP was completely inhibited by NaN_3 (0.1%) or KCN (1 mM) as shown in Fig. 1.

Table 3. Susceptibility to KM in various media

Medium	MIC to KM		
	K-Ps 102	K-Ps 47 RFP	K-Ps 47 RFP (kR 102)
Tryptosoy	1,600	100	1,600
Low phosphate	400	25	400
Low phosphate +$MgCl_2$(0.1 M)	3,200	100	1,600

It is known that low phosphate media increase the incorporation of drugs into bacterial cells.[8] An overnight culture in Tryptosoy liquid medium was inoculated into low phosphate media containing various concentrations of KM to determine MIC's (minimum inhibitory concentrations). The MIC of KM for K-Ps 47 RFP is 25 mcg/ml, and the MIC for K-Ps 102 and K-Ps 47 RFP (kR102) was 400 mcg/ml: all the MIC's were one-fourth of those determined in Tryptosoy liquid medium as shown in Table 3. However, in low phosphate medium with added $MgCl_2$ such decrease of MIC was not observed with any strain, but MIC values were similar to those in Tryptosoy liquid medium.

Though no data are shown, the sensitivity of K-Ps 47 RFP (kR102) to KM was partially increased by EDTA to change the structure of outer membrane and by carbenicillin and glycine to break mucopeptide, but it still retained KM resistance. Therefore, it is thought that the main KM resistance mechanism of K-Ps 47 RFP (kR102) is that the active transport system relating to the respiratory enzyme system in the cytoplasmic membrane (inner membrane)[9] does not function normally, making it difficult for KM to reach the inner membrane region, or even if it reaches the inner membrane, KM does not reach the ribosome, because the active transport system does not function normally because of lowered affinity between the active transport system and KM.

There are a number of reports[2~4,10~14] dealing with the decreased permeability of TC, CP, SM, gentamicin (GM) *etc.* into bacterial cells based on a change of cell envelope. In TC resistant R^+ *Pseudomonas aeruginosa*, a specific protein for TC resistance has been detected,[15] and its relation with TC permeability in cell envelope is considered. BRYAN *et al.*[16] presented the opinion

that the resistance mechanism of GM resistant *Pseudomonas aeruginosa* strain having nontransferrable plasmid DNA was caused by the lowered affinity for GM in the GM transport system, or the decrease of permeable access. However, they did not make a comparison with the same host strain which does not have plasmid DNA. Our experiments strongly suggest that the presence of R plasmid affects the cell envelope directly.

In summary, the conjugant strain K-Ps 47 RFP (kR102) did not produce any KM inactivating enzymes (phosphorylating, adenylylating, and acetylating), and its ribosome was sensitive to KM. Therefore, it was suggested that the KM resistance mechanism of the R plasmid, kR 102, involves some change in the structure or function of the cell envelope.

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