MECHANISM OF CHLORAMPHENICOL-RESISTANCE MEDIATED BY kR102 FACTOR IN *PSEUDOMONAS AERUGINOSA*

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The chloramphenicol (CP)-resistance mechanism of five-drug-resistant R factor (kR102) of *Pseudomonas aeruginosa* K-Ps 102 derived from a clinical specimen was investigated. Neither inactivation by acetyltransferase of CP nor induced resistance by CP was recognized. Reduced affinity of the ribosome to the drug was not seen in the result of incorporation experiment of ^{14}C-valine by phage f2 RNA and ribosome of K-Ps 102. However, on spheroplasts by glycine treatment, remarkable increase of CP susceptibility was observed. From the above evidence, it was considered that the CP-resistance barrier controlled by kR102 factor would be in the cell wall and the surface layer of cytoplasma and that the mechanism of CP-resistance was possibly by decreased membrane permeability of CP. However, the susceptibility to CP of the susceptible strain still increased by the formation of spheroplasts. Consequently, it was considered that R factor might be controlling the function of membrane permeability of the cells.

The mechanism of chloramphenicol (CP) resistance has been shown to be due to CP-inactivation by the CP acetyltransferase (CATase) in *Staphylococci* having CP-resistant plasmid[1] or in enteric bacteria having R factor[2]. However, as to some *Escherichia coli* being resistant owing to R factor, decrease of membrane permeability of CP is reported to be the mechanism of increased resistance.[3]

Recently, R factor has been found in *Pseudomonas aeruginosa*, but there is not yet any report on the mechanism of CP-resistance controlled by R factor of *P. aeruginosa*. This paper deals with the mechanism of CP-resistance controlled by kR102 factor derived from *P. aeruginosa* strain, K-Ps 102.

Materials and Methods

Strains used: We used *P. aeruginosa* strains, K-Ps 102, having R factor (kR102) resistant to five drugs; tetracycline (TC), CP, streptomycin (SM), sulfonamide (SA), and kanamycin (KM); rifampicin (RFP) resistant mutant (K-Ps 47 RFP) of sensitive *P. aeruginosa* strain K-Ps 47, and the conjugant strain K-Ps 47 RFP (kR102) obtained from K-Ps 47 RFP by mating with K-Ps 102.[4] *E. coli* K-12 W3110 RFP (R100-1), which was an RFP resistant mutant of *E. coli* W3110 having R100-1 factor[2] (resistant to TC, CP, SM and SA), was employed as a reference strain for CP-inactivation.

Media: These studies were conducted using brain heart infusion broth (Difco: BHI) containing 0.4 % KNO_3 (K-BHI) for the CP-pretreatment experiment, medium B (nutrient broth: Eiken) for preparation of the crude enzyme, and tryptone medium (10 g of Polypeptone, 8 g of NaCl, 1 g of yeast extract powder, 1 g of glucose and 2 ml of 1 M $CaCl_2$ in 1 liter; pH 7.2) for preparation of ribosomes and for multiplication of phage f2. For spheroplast formation, medium BS (medium B containing 12 % saccharose) and medium BGS (medium BS containing 3 % glycine) were used. Agar was added to them, if necessary, to use them as a solid medium.

Determination of minimum inhibitory concentration (MIC): An overnight culture of bacteria in peptone water was diluted 100 times, and a platinum loop of the dilution was inoculated to nutrient agar plates containing various concentrations of each drug. After culturing overnight at 37°C, the MIC was determined. MIC on CP-pretreatment was determined similarly on the bacteria cultured overnight with peptone water containing various concentrations of CP lower than MIC.

Determination of 50 % growth inhibition dose (ID_{50}): Each 0.1 ml of bacterial culture in the exponential growth phase was added to 10 ml of K-BHI containing various concentrations of CP, and the growth curve was recorded for 6 hours by Jouan-Biophotometer (Paris). From the growth % in the exponential phase, ID_{50} was obtained according to TREFFER's method.[5] ID_{50} on CP-pretreatment was similarly determined on the bacteria cultured for 2 hours in K-BHI containing various concentrations of CP less than MIC.

Determination of CP-inactivation: The reaction was carried out according to the system of enzymatic inactivation of aminoglycoside antibiotics reported previously,[6] except that it was performed at 37°C for 18 hours and the residual potency of the antibiotic after the reaction was determined with *Sarcina lutea* ATCC 9341.

Determination of chloramphenicol acetyltransferase (CATase) activity: The activity was determined according to the method reported previously,[2] as using acetyl CoA and dithiobisnitrobenzoic acid (DTN). Protein content was determined according to the method of LOWRY *et al.*[7]

Formation of spheroplasts: To 1 ml of the overnight culture in medium B, 9 ml of fresh medium B was added, and cultured to obtain 0.5 OD at 600 nm; to 1 ml of the culture 9 ml of medium BGS was added and subjected to shaking culture for 3 hours. After confirming formation of spheroplasts, the culture was diluted adequately with medium BS or sterilized water, spread onto medium BS-agar and medium B-agar, and incubated overnight at 37°C. The number of bacteria was counted and the number of spheroplasts was obtained by subtracting the number of colonies on the medium B agar (colonies of which were not converted to spheroplasts) from the number of colonies on the medium BS-agar (total colonies).[8]

Determination of incorporation of ^{14}C-valine: S-105 fraction and ribosomes were prepared according to the method of MODOLLEL and DAVIS,[9] and phage f2 RNA was prepared according to the method of GESTLAND.[10] The reaction was carried out according to the method of TSENG *et al.*[11] with the following reaction mixture: 60 mM Tris buffer, pH 7.8; 30 mM NH_4Cl; 10 mM Mg-acetate; 10 mM reduced glutathione; 1 mM adenosine triphosphate (ATP); 0.2 mM guanosine triphosphate (GTP); 5 mM creatine phosphate; 50 mcg creatine kinase (Sigma)/ml; 0.05 mM amino acids of 19 kinds other than valine; 0.03 mM ^{14}C-valine (225 mCi/mmole, Daiichi Kagaku); 0.1 mg phage f2 RNA/ml; 1 mg *E. coli* W, t-RNA/ml (Sigma); 0.2 volume of S-105 fraction and 0.2 volume of ribosome suspension (10 mg/ml); and adequate amount of CP.

All the above materials except f2-RNA and ^{14}C-valine were mixed, and after allowing to stand at 34°C for 10 minutes, the additional two materials were added to react at the same temperature for 30 minutes. Fifty μl of the reaction mixture was removed, and after adding 0.5 ml of cold 5 % trichloroacetic acid (TCA), the mixture was heated at 90°C for 15 minutes, filtered through membrane filter (Toyo Roshi Ltd; TM-2, 0.45μ; size, 13 m/m), and rinsed four times repeatedly with 1 ml of 5 % TCA. ^{14}C-Valine on the filter was determined by a liquid scintillation counter of Packard 3330 Type. The solvent for the liquid scintillation was prepared with a base of toluene (5 g of 2, 5-diphenyloxazol (POP), 0.3 g of dimethyl-POPOP, and 1 liter of toluene).

Results

CP Sensitivity and its Induced-Resistance by the Drug

MIC values of *P. aeruginosa* strains revealed that CP susceptibility was not different between the donor strain having R factor and the conjugant strain which accepted the R factor.

Increase of the resistance by CP pretreatment was not observed by determining MIC and in the comparison of ID_{50} value which is considered to be the most sensitive to the change of the susceptibility for antibiotics. Table 1 shows the results when using the concentrations of 25 mcg/ml with a CP-resistant strain and of 6.25 mcg/ml with a CP-susceptible strain on CP pretreatment. Though the data are not shown here, induction of CP-resistance on pretreatment with 25 mcg of CP/ml was not recognized with the other five strains of *P. aeruginosa* having CP-resistant R factors reported previously.[4]

Table 1. Comparison of chloramphenicol(CP) resistance of *P. aeruginosa* pretreated with or without CP

Strain	MIC		ID_{50}	
	−	+	−	+*
K-Ps 102	1,600	1,600	50	60
K-Ps 47 RFP	100	50**	4.2	3.1**
K-Ps 47 RFP (kR 102)	1,600	1,600	56	49

* Pretreatment with 25 mcg of CP/ml, yes(+), not(−)
** Pretreatment with 1.6 mcg of CP/ml

Mechanism of CP-resistance Controlled by R Factor, kR102

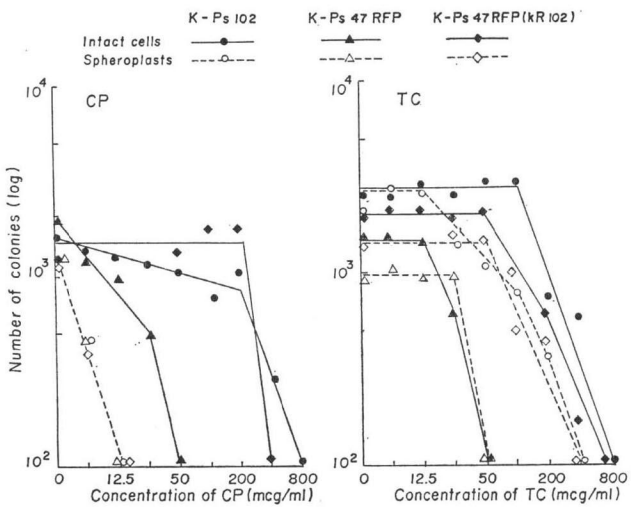
The test strains were ruptured by means of supersonic oscillation, and the supernatant ($30,000\times g$) thus obtained was used to examine CP inactivation in the presence of ATP, CoA, and Mg-acetate, or in the presence of acetyl-CoA and DTN. The results are shown in Table 2. As can be seen in the table, only the reference strain of *E. coli* W3110 RFP (R100-1) having CATase activity showed CP-inactivation in every determination, but three other *P. aeruginosa* strains could not inactivate CP. CP inactivation with intact cells of the above strains in the presence of 50 mcg/ml

Table 2. Inactivation of chloramphenicol(CP) and the CP-acetyltransferase (CATase) activity by crude extracts from various strains

Crude extract from	Inactivation	Specific enzyme activity* (units/mg of protein)
K-Ps 102	−	<0.0005
K-Ps 47 RFP	−	<0.0005
K-Ps 47 RFP (kR102)	−	<0.0005
W3110 RFP (R100-1)	+	1.30

* Acetylation of 1μ mole of CP/min/mg of protein

Fig. 1. Chloramphenicol(CP) and tetracycline(TC) resistance of either intact cells or spheroplast of *P. aeruginosa* strains



of CP was also not recognized

Then, the CP susceptibility of spheroplasts of the strain by glycine treatment was examined and simultaneously TC susceptibility was observed[12] (Fig. 1). The TC susceptibility of K-Ps 102 and K-Ps 47 RFP (kR102) increased up to 400 mcg/ml, while CP susceptibility was observed to increase greatly; it increased up to 12.5 mcg/ml in K-Ps 47 RFP, and each strain was also sensitized up to the same level.

Ribosomes were extracted from each strain and the CP susceptibility was observed and the results were shown in Table 3. There had been no report on determination of the CP susceptibility with the ribosome of *P. aeruginosa* having R factor. The strains used in the experiment were all susceptible to CP, and the degree of susceptibility was almost similar both in the resistant strains and susceptible strains.

Table 3. Inhibition of amino acid incorporation by chloramphenicol in cell-free systems obtained from *P. aeruginosa* strains

Strain*	Concentration of CP (mcg/ml)	Incorporation of ^{14}C-valine (cpm)**
K-Ps 102	0	1134
	20	147
	50	0
K-Ps 47 RFP	0	933
	20	168
	50	5
K-Ps 47 RFP (kR102)	0	907
	20	226
	50	9

* The strains were used as a source of the ribosomes or S-105 fractions which were prepared as described in Materials and Methods.

** Background counts per minute were 105 on an average.

Discussion

As described in the previous report, kR102 factor is an R factor controlling five drug-resistances, and the mechanism of the TC-resistance is owing to decrease of TC-incorporation into cells.[12] However, since inactivation in CP-resistance, SM-resistance, and KM-resistance, in which inactivation of a drug is thought to be a mechanism of resistance, is not shown by kR102 factor, it is considered to be an R factor having a special resistance mechanism. The kR102 factor is found in *P. aeruginosa*, and can be transferred to *P. chlororaphis*, *P. putrefaciens*, and *P. pseudoalcaligenes* among *Pseudomonas* genus, but transfer to *E. coli* has not been recognized. Ribosomes of K-Ps 102 are known to exhibit SM-resistance (unpublished data). And it has been reported that most of *P. aeruginosa* strains showed low levels of CATase activity, which, however, did not appear sufficient to explain their resistance.[13] We also tried to detect CATase as to the strains used, according to the method of OKAMOTO *et al.* using $CH_3{}^{14}COONa$, ATP, CoA, each crude extract and CP. From the results, 3-O-monoacetyl CP and 1, 3-O-diacetyl CP were found only in the reference strain (*E. coli* W3110 RFP (R100-1)). However, these inactivated products were not observed in K-Ps 102, K-Ps 47 RFP and K-Ps 47 RFP (kR102) (unpublished data).

Therefore, as described in this report, it is revealed that CP-resistance by kR 102 factor is not due to inactivation by acetylation nor due to resistance of ribosome, but the mechanism of resistance development is decrease of permeability of CP into cells. A similar R factor having such a special resistance mechanism is R70 factor of *E. coli* reported by NAGAI and MITSUHASHI.[8] R70 factor was reported to show induction of resistance by CP, but it is quite different in kR102, as induction of CP-resistance was not seen in the latter. Increase of sensitivity to CP of spheroplasts was specific to CP, and it seemed to be a factor as barrier of sensitivity between cell wall and cell membrane, since it was greatly changed by injury of the cell wall. In both of the donor of R factor and the resistant strain receiving R factor, the behaviors of the sensitivities of the intact cells and the spheroplasts were quite the same, and also the sensitive strain K-Ps 47 RFP increased its sensitivity in spheroplasts up to the same degree to that of R^+ strain:

it suggest the existence of ultra-sensitive strain having the sensitivity higher than that of K-Ps 47 RFP. Such an ultra-sensitive strain (MIC: less than 25 mcg/ml of CP) was found in 2% of the strains examined. However, the growth of these strains was slightly observed on overnight cultivation in the broth containing 25 mcg of CP/ml, and the MIC of the culture became the same to that of K-Ps 47 RFP. The phenomenon has not been further discussed. The difference of the sensitivity between the spheroplasts and the intact cells of K-Ps 47 RFP should suggest the existence of barrier in the cell wall, which is thought as a resistance mechanism of kR102 factor. Accordingly, the resistance mechanism of R factor might be qualitative or quantitative control of the barrier, which is easily separated from the cell surface on the formation of spheroplasts. R factor whose resistance mechanism is not inactivation should be noticed as well as R70 factor reported by NAGAI and MITSUHASHI.[3]

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