LABILITY OF RESISTANCE TO AMINOGLYCOSIDE ANTIBIOTICS IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The determinants for resistance to aminoglycoside antibiotics in some strains of *Pseudomonas aeruginosa* were irreversively lost during storage or by treatment with ethidium bromide. This fact has indicated the presence of plasmids governing resistance to aminoglycosides in the pseudomonads as well as R factor in gram-negative enteric bacteria and plasmid in staphylococci.

Many papers have described the extrachromosomal determinants governing drug resistance in gram-negative enteric bacteria[1] and in staphylococci[2,3,4], which are irreversibly lost from the host strains or conjugally transmissible by cell-to-cell contact This paper deals with the elimination of the determinants governing resistance to aminoglycoside antibiotics in some strains of *Pseudomonas aeruginosa* isolated from clinical specimens.

MATERIALS AND METHODS

Bacterial strains. The strains of *Pseudomonas aeruginosa* used in this experiment were collected and stocked in cooked-meat medium.

They were isolated from clinical specimens by this laboratory or by many participating laboratories in a research project in Japan: "Studies on the drug resistance in gram-negative bacteria" (Chief, S. ISHIYAMA, Nihon University, Tokyo). They were isolated from patients, purified and then sent to this laboratory. This laboratory spread each strain on HI agar plate and picked one colony for stock culture. After examining drug resistance and biological properties, single colony of each strain was stocked in cooked-meat medium.

Media. Heart infusion broth (HI; Eiken, Tokyo) and HI agar were used routinely. Cooked-meat medium (Difco) was used for stock culture.

Drug resistance. Drug resistance was examined on agar plates containing serial two-fold dilutions of each drug. After overnight incubation in HI broth, a loopful of 100-fold diluted culture was plated on HI agar plates containing each drug. Minimum inhibitory concentration (MIC) of drug was scored after 18 hr. incubation at 37°C.

Loss of drug resistance by treatment with ethidium bromide. One tenth ml of overnight HI culture of each strain was inoculated in 1 ml broth containing ethylenediaminetetraacetic acid (EDTA) (10^{-3} M/ml) and incubated at 37°C. Four cultures were prepared for each strain.

After 3 hr. of incubation, the cultures were centrifuged at 3,000×g for 10 min. and 1 ml of fresh broth containing each 25, 50, 100 and 200 mcg/ml of ethidium bromide (EB) was added to the precipitates, respectively. After incubation for 18 hr. at 37°C, a culture that showed visible growth in the highest concentration of EB, was spread on HI agar plates and used as the master plates for the replica-plating method. The master plates of each strain were replica-plated on HI agar plates containing each streptomycin (50 mcg/ml), kanamycin A (50 mcg/ml), 3′, 4′-dideoxykanamycin B (12.5 mcg/ml), lividomycin (50 mcg/ml), and gentamicin C (12.5 mcg/ml). Drug resistance of the colonies from which drug resistance was found to be lost by this method, was determined by the agar dilution method.

Loss of drug resistance after storage in cooked-meat medium. After 3 successive single colony isolations on HI agar plate, each strain was stocked in cooked-meat medium in the cold. Each strain was spread on HI agar plate after 20 days' storage and used as the master plates for the replica-plating method. Loss of resistance was examined by the same method desribed above. Elimination of drug resistance was defined by 4-fold or more decrease in the level of resistance than that of original one.

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Drugs. Streptomycin (SM), kanamycin A (KM-A), kanamycin B (KM-B), and 3′, 4′-dideoxykanamycin B (DKB) were supplied by H. UMEZAWA. Lividomycin (LV) was given by Kowa Co., Tokyo. Gentamicin C (GM) complex was supplied by M. J. WEINSTEIN.

RESULTS

Antibacterial activity of aminoglycoside antibiotics toward *P. aeruginosa*. The antibacterial activity of various aminoglycoside antibiotics against *P. aeruginosa* was investigated by using 645 clinical isolates. As shown in Fig. 1, the antibacterial activity of DKB toward *P. aeruginosa* was the highest and followed by GM, LV, SM, KM-B and KM-A. GM and DKB were found to be quite effective against *P. aeruginosa*.

Loss of resistance after storage. One hundred and eighty strains of *P. aeruginosa* were randomly picked from our stock cultures and subjected to 3 successive single colony isolations. One colony of each strain was picked and inoculated in HI broth. After determination of drug resistance, each strain was stocked in cooked-meat medium. After 20 days' storage in refrigerator, loss of resistance was examined by the method described in Materials and Methods. As shown in Table 1, 21% of the stock cultures lost their resistance to one of these aminoglycosides and some of them lost jointly double or triple resistance. To confirm this finding, 10 strains were selected randomly and the lability of their resistance was examined by treatment with ethidium bromide. As shown in Table 2, 3 strains lost SM, KM, and GM resistance and 4 strains lost jointly (KM, LV), (GM, SM), and (KM, GM) resistance.

Table 1. Loss of drug resistance in *P. aeruginosa* during storage

Loss of resistance to	No. of strains
SM	6
KM	17
GM	6
KM, SM	2
KM, GM	4
KM, LV	2
GM, SM	1
KM. SM. LV	1
Total 180	39 (21%)

One hundred and eighty strains of *P. aeruginosa* were stocked in cooked-meat medium and their resistance was examined after 20 days of storage in refrigerator.

DISCUSSION

We found by chance that multiple resistance in *Shigella* strains was lost at a high frequency during

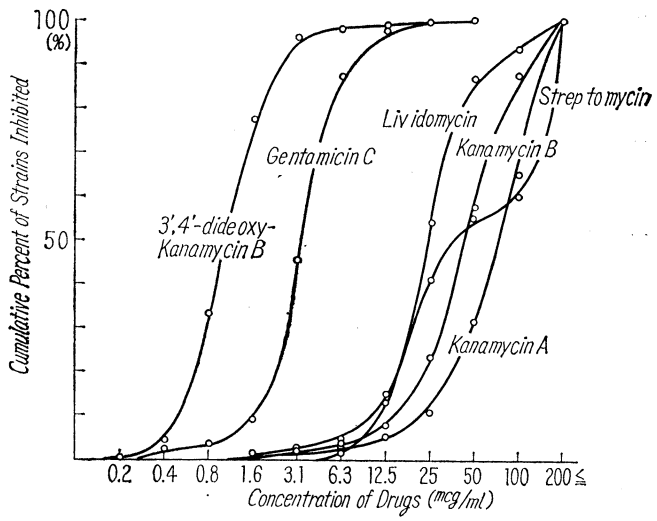
Table 2. Loss of resistance in *P. aeruginosa* by treatment with ethidium bromide

Strain No.	MIC (mcg/ml) [a]	No. of colonies [b] examined
348	SM >800 (12.5)	12/351
573	>800 (50.0)	14/340
394	MK 1,600 (25.0)	10/250
166	1,600 (50.0)	21/260
2,476	GM 400 (6.3)	21/380
1,301	200 (3.1)	19/240
TI-13	KM 1,600 (12.5)	8/280
	LV 1,600 (12.5)	
375	KM 1,600 (12.5)	9/230
	LV 1,600 (12.5)	
907	GM 200 (3.1)	4/380
	SM >800 (12.5)	
137	KM >800 (50.0)	6/280
	GM 400 (12.5)	

[a] Bracketed numbers indicate the MIC of the colonies from which drug resistance was lost after treatment with ethidium bromide.

[b] Numerator indicates the number of colonies from which drug resistance was lost. Denominator indicates the total number of colonies examined.

Fig. 1. Antibacterial activity of various aminoglycosides against *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC) of aminoglycosides toward 645 strains of *P. aeruginosa* was examined.



storage in cooked-meat medium[5]. In addition to this finding, artificial elimination of drug resistance[6], transmission of resistance regardless of the polarity of F factor[7] and interruption of the transmission of resistance by blender treatment[8] established the presence of R factor in gram-negative enteric bacteria[1].

The first results of irreversible elimination of resistance to macrolide (Mac) antibiotics led us to the conclusion that the determinant responsible for Mac resistance in staphylococci is located extrachromosomally, probably being on a plasmid[2]. According to transduction and genetic investigation, it was concluded that there are two plasmids ; one carrying a penicillin (PC) resistance determinant and another harboring both PC- and Mac-resistance determinants[4,9,10].

According to the isolation of the recombination-deficient (*rec*$^-$) mutants and transduction of labile resistance to *rec*$^-$ recipients, many resistance determinants in staphylococci have been concluded to be located on plasmids[3].

LOWBURY *et al*[11]. Eliminated high level carbenicillin resistance in some strains of *P. aeruginosa*, suggesting that the resistance determinant is located extrachromosomally. Our findings also indicated that some determinants governing aminoglycosides resistance in the pseudomonads were irreversibly lost during storage or by treatment with ethidium bromide.

Previous papers[1] reported the wide distribution of R factors among the most genera of the *Enterobacteriaceae*. On the other hand, we have not conclusively demonstrated thus far the R factors in *P. aeruginosa* which are conjugally transmissible. SMITH and ARMOUR[12] described the presence of R factor carrying KM resistance in *P. aeruginosa*. Recent papers also reported the presence of R factors in the pseudomonads, which could mediate PC resistance (β-lactamase production)[13] and (TC, KM, PC)-resistance[14], although there still remains the possibility that this transmission may be caused by transduction with phage lysates. Therefore, we are still now incapable of drawing conclusion whether the labile resistance determinants in the pseudomonads are of R-factor type or of staphylococcal plasmid type that is nontransmissible by conjugation.

We have described the thought that transduction with phage lysates from lysogenic strains is the main factor for rapid acquisition and wide distribution of multiple resistance in staphylococci[3,15]. The fact that most of the pseudomonads are lysogenic, exhibits a similar situation to that in staphylococci. The genetic analysis of the extrachromosomal resistance determinants in *P. aeruginosa* is now in progress and will be described elsewhere.

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