ISOLATION OF TWO TYPES OF *PSEUDOMONAS AERUGINOSA* MUTANTS HIGHLY SENSITIVE TO A SPECIFIC GROUP OF BETA-LACTAM ANTIBIOTICS AND WITH DEFECT IN PENICILLIN-BINDING PROTEINS

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Two types of mutants highly sensitive to beta-lactam antibiotics were obtained from *Pseudomonas aeruginosa* PAO 2142 by treatment with N-methyl-N′-nitro-N-nitrosoguanidine. One type of mutant showed over 30 times higher sensitivity to mecillinam, carbenicillin and sulbenicillin than did the parent, but not to most other beta-lactam antibiotics tested. In contrast, the other type mutant was about 30 times more sensitive to ampicillin, cephaloridine, cefoxitin and cefmetazole, but resistant to mecillinam, carbenicillin and sulbenicillin at the same level as the parent. Beta-lactamase activity of these mutants was not different from that of the parent. Defect in either of penicillin-binding proteins 1A/1B or 5 was observed in some mutants of *P. aeruginosa* highly sensitive to beta-lactam antibiotics.

Pseudomonas aeruginosa induces infection extremely refractory to chemotherapy because it is resistant to many chemotherapeutic drugs including beta-lactam antibiotics. It has been reported for *P. aeruginosa*, as well as in other species of microorganisms, that the production of beta-lactamase and mutation in constituents of outer membrane have resulted in drug resistance.[1,2]

It is also known for *P. aeruginosa* that beta-lactam antibiotics exert their bacteriostatic and bactericidal action against bacterial cells as a result of covalently binding to the enzyme(s) involved in transpeptidation of newly synthesized peptidoglycan and inhibiting their activities.[3,4] Therefore, target(s) of beta-lactam antibiotics can be detected as penicillin-binding protein(s): PBP(s). In *Escherichia coli*, mutants with defects in some PBP(s) have already been selected among temperature-sensitive mutants (ts-mutants), from which the physiological function and enzymatic activity of PBPs have been disclosed,[5~10] and the defect in PBP-1Bs has been known to result in supersensitivity to beta-lactam antibiotics.[6] In *P. aeruginosa*, six major and several minor PBPs have been detected and have been shown to be species-specific,[11,12] but their function remains to be elucidated.

Taking focus on target proteins, the authors attempted to obtain mutants of *P. aeruginosa* highly sensitive to beta-lactam antibiotics to clarify the mechanism of drug resistance for beta-lactam antibiotics and the physiological function of PBPs. To efficiently obtain strains with mutations in target proteins (PBPs), we mainly used indirect methods for selecting mutants highly sensitive to beta-lactam antibiotics; that is, first we selected temperature-sensitive mutants (ts-mutants) by replica method and secondly chose the mutants highly sensitive to beta-lactam antibiotics among ts-mutants.

Materials and Methods

Drugs used

The sources of the beta-lactam antibiotics used in this work were as follows: penicillin G potassium salt and ampicillin from Banyu Pharmaceuticals Co. (Tokyo); apalcillin from Sumitomo Chemical Co. (Osaka); carbenicillin and cloxacillin from Fujisawa Pharmaceutical Co. (Osaka); piperacillin from Toyama Chemical Co. (Tokyo); sulbenicillin, mecillinam and cefsulodin from Takeda Chemical Co. (Tokyo); 6-aminopenicillanic acid from Sigma; cephaloridine from Torii Pharmaceutical Co. (Tokyo); cephaloglycin from Shionogi & Co. (Osaka), cefoxitin from Daiichi Seiyaku Co. (Tokyo); cefmetazole from Sankyo Co. (Tokyo); 7-aminocephalosporanic acid and 7-aminodesacetoxycephalosporanic acid from Antibioticos (Spain). [^{14}C]Penicillin G potassium salt (specific activity 54 Ci/mol) was purchased from the Radiochemical Centre (Amersham, England).

Strains used

Pseudomonas aeruginosa PAO 2142 was kindly given to us by E. MATSUMOTO at Shinshu University (Matsumoto, Japan). This strain is a derivative from *P. aeruginosa* PAO 1 and shows an auxotrophy for isoleucine-valine, lysine and methionine.

Isolation of hs-mutants

Mutants of *P. aeruginosa* PAO 2142 which were highly sensitive to beta-lactam antibiotics were obtained from shaken cultures developed in nutrient broth (Nissui, Tokyo) at 30°C and harvested at OD_{650}=0.5 to 0.6. The cells were mutagenized with 100 mcg/ml of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; Nakarai Chemical Co., Kyoto) in nutrient broth (pH 7.0) for 1 hour at 30°C and then washed twice with fresh nutrient broth. After further shaking culture in fresh nutrient broth for 3 hours at 30°C, the cells were packed by centrifugation, appropriately diluted with saline and inoculated onto the plate of heart infusion agar (HIA; Nissui, Tokyo). The colonies formed after incubation overnight at 30°C were replicated onto two plates of HIA. One plate is incubated at 30°C and the other at 44°C. Ts mutants which have colony-forming ability at 30°C but not at 44°C were subjected to drug sensitivity against beta-lactam antibiotics and highly sensitive mutants (hs-mutants) were selected. In addition to the above mentioned method, we also obtained beta-lactam highly sensitive mutants (hs-mutants) directly by replicating the HIA plate with 2 mcg/ml of carbenicillin or 100 mcg/ml of cefoxitin after mutagenization with MNNG (direct method for obtaining hs-mutants).

Isolation of bla$^-$-mutants

Beta-lactamase-less mutants (bla$^-$-mutants) were obtained from *P. aeruginosa* PAO 2142 as follows. Strain PAO 2142 was treated with 100 mcg/ml of MNNG in nutrient broth (pH 7.0) for 1 hour at 30°C and washed with saline. After an appropriate dilution, 0.1 ml of the cell suspension forming 100 to 200 colonies per plate was spread on Difco nutrient agar plates. The colonies formed after incubation overnight at 30°C were overlayed with soft agar containing 5 ml of 1.2% Difco agar, 1 ml of 10 mg/ml KI and 4 mg/ml iodine, 0.5 ml of 0.5% soluble starch and 0.5 ml of 10% penicillin G (PC-G). Constitutive mutants decolorized the purple color of iodine-starch complex, as a result of the hydrolysis of PC-G, and formed large halos around them. One of the constitutive mutants, C-1-2, was further mutagenized and selected by the above mentioned method. Colonies which did not form halos around them on agar containing iodine, starch and PC-G were picked and purified and referred to as bla$^-$-mutants. One of them is mutant bla$^-$-5.

MIC determination

Minimal inhibitory concentration(MIC) values were determined by serial two-fold agar dilution on HIA. The 5 μl of cell suspension with 10^8 colony forming units per milliliter was inoculated onto agar plates containing the drug tested with Microplantor (Sakuma Seisakujo; Tokyo).

Detection of penicillin-binding proteins (PBPs)

Membrane preparation and detection on PBPs were done according to the method reported previously[12].

Measurement of beta-lactamase activity

Beta-lactamase activity was assayed by both bioassay[13] and spectrophotometry[14].

Phage sensitivity

Phage sensitivity was done according to SAKAMOTO *et al.*[15]

Protein estimation

Protein concentration was determined by LOWRY's method[16].

Results and Discussion

The MICs against 17 beta-lactam antibiotics of representative mutants highly sensitive to beta-lactam antibiotics(hs-mutants) are shown in Table 1. Mutants hs-28, 86 and 233 were obtained by a direct method, and hs-208, 246 and 257 by an indirect method(data for hs-28 and hs-246 not shown in Table 1). Considering the sensitivity for beta-lactam antibiotics, mutants obtained were classified into two types. Mutant hs-86 and 233, which were selected as strains sensitive to 2 mcg/ml of carbenicillin,

Table 1. Drug sensitivity to beta-lactam antibiotics of highly sensitive mutants of *Pseudomonas aeruginosa* PAO 2142.

Drugs		Minimal inhibitory concentration (mcg/ml)*					
		Parent PAO 2142	Mutants				bla⁻-5
			Type I		Type II		
			hs-86	hs-233	hs-208	hs-257	
Penicillins	6-APA	800	400	400	200	200	100
	PC-G	3,200	800	1,600	50	50	25
	ABPC	400	200	200	12.5	3.13	12.5
	APPC	1.56	0.1	0.2	0.78	0.2	0.39
	PIPC	3.13	0.39	0.39	3.13	0.39	0.78
	MCIPC	3,200	200	100	3,200	800	3,200
	CBPC	25	0.39	0.39	25	3.13	12.5
	SBPC	12.5	0.2	0.39	12.5	3.13	6.25
	TIPC	6.25	0.2	0.39	6.25	1.56	ND
Amidinopenicillin	MPC	200	1.56	1.56	100	400	100
Cephalosporins	7-ACA	>3,200	800	1,600	>3,200	3,200	>3,200
	7-ADCA	>3,200	>3,200	>3,200	>3,200	>3,200	ND
	CEG	>3,200	1,600	3,200	800	200	400
	CER	>3,200	>3,200	3,200	25	50	25
	CFS	0.78	0.2	0.2	0.78	0.2	0.39
Cephamycins	CFX	800	800	800	25	12.5	25
	CMZ	1,600	1,600	1,600	50	25	50

Abbreviation: 6-APA, 6-aminopenicillanic acid; PC-G, penicillin G; ABPC, ampicillin; APPC, apalcillin; PIPC, piperacillin; MCIPC, cloxacillin; CBPC, carbenicillin; SBPC, sulbenicillin; TIPC, ticarcillin; MPC, mecillinam; 7-ACA, 7-aminocephalosporanic acid; 7-ADCA, 7-aminodesacetoxycephalosporanic acid; CEG, cephaloglycin; CER, cephaloridine; CFS, cefsulodin; CFX, cefoxitin, CMZ, cefmetazole; ND, not done.

* Minimal inhibitory concentration was determined by a serial two-fold agar dilution method. Five μl of cell suspension (10^6 CFU/ml) was inoculated onto heart infusion agar containing an appropriate concentration of drug and incubated for 20 hours at 30°C. Mutants hs-28 and hs-246 showed patterns of drug sensitivity similar to those of hs-208 and hs-257, although data are not shown.

showed 128 times higher sensitivity to mecillinam (MPC), 32~64 times to carbenicillin(CBPC), sulbenicillin(SBPC), 16~32 times to ticarcillin(TIPC) and cloxacillin(MCIPC), 8~16 times to apalcillin(APPC) and piperacillin(PIPC) compared with that of the parent. They(type I) showed almost the same sensitivity as the parent to penicillin G (PC-G), cephaloridine(CER), cefoxitin(CFX) and cefmetazole(CMZ). In contrast, mutants selected as a strain sensitive to at least one of 17 beta-lactam antibiotics tested among temperature-sensitive mutants(ts-mutants), for example hs-208, 246 and 257 (data for hs-246 not shown), showed more than 64 times higher sensitivity to CER, 32~128 times to ABPC, 32~64 times to PC-G and 16~64 times to CFX and CMZ compared with that of the parent. The sensitivity of these mutants(type II) to MPC, CBPC, SBPC and TIPC was almost the same as that of the parent. Mutant hs-28, which was directly obtained on the plate with 100 mcg/ml of CFX, showed MICs similar to type II of the mutants(data not shown). Beta-lactam antibiotics to which type I mutants show high sensitivity were different from those for type II mutants.

Beta-lactamase less mutant bla$^-$-5, obtained by the other method, showed MIC patterns similar to type II mutants.

Table 2. Beta-lactamase activity of *Pseudomonas aeruginosa* mutants highly sensitive to beta-lactam antibiotics.*

	Strain	Specific activity (U/mg of protein)	
		Not induced	Induced**
Parent	PAO 2142	6.8×10^{-4}	2.5×10^{-2}
hs-mutants I	hs-86	11.0×10^{-4}	8.3×10^{-2}
	hs-233	8.3×10^{-4}	2.4×10^{-2}
hs-mutants II	hs-28	9.4×10^{-4}	8.5×10^{-4}
	hs-208	5.7×10^{-4}	1.4×10^{-2}
	hs-246	6.7×10^{-4}	7.2×10^{-2}
	hs-257	5.3×10^{-4}	2.4×10^{-2}
bla$^-$-mutants	bla$^-$-5	$<3.3\times10^{-4}$	$<5.2\times10^{-4}$

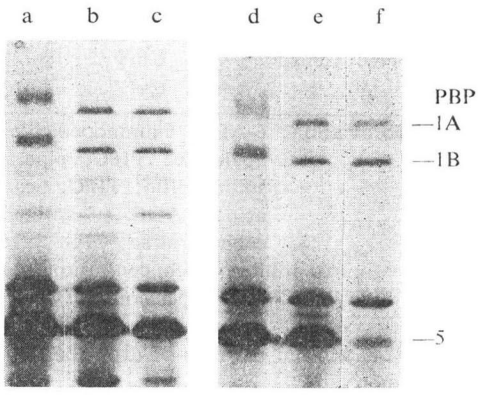
* Beta-lactamase activity was determined by bioassay method. One tenth milliliter of appropriately diluted enzyme preparation was added to 1 ml of 500 μM cephaloridine in 100 mM phosphate buffer (pH 7.0) and then incubated at 30°C. At an appropriate time (2~120 minutes), the reaction was stopped by addition of 2 ml of ethanol. The mixture was bioassayed on the agar plate seeded with *B. subtilis* ATCC 6633. Activity (unit; U) was expressed as μmole of cephaloridine degraded per minute at 30°C. Almost the same result was obtained by spectrophotometric method using 100 μM of cephaloridine as a substrate.

** At OD_{650} of 0.3 to 0.5, penicillin G was added to culture to a final concentration of 2 mg/ml and further incubated for 3 hours at 30°C.

Intracellular beta-lactamase is known to play an important role in resistance to beta-lactam antibiotics, as a result of the fact that lack of production and inducibility of beta-lactamase has rendered cultures highly sensitive[2]. Also in this experiment, beta-lactamase-less mutant bla$^-$-5, with a very low level of beta-lactamase activity (less than 5×10^{-4} U/mg of protein), was con-

Fig. 1. Penicillin-binding proteins of *P. aeruginosa* PAO 2142 and its mutants.

Membrane preparations were incubated for 10 minutes at 30°C (a~c) or 40°C (d~f) and then further incubated with 30 mcg/ml of [^{14}C]-penicillin G(PC-G) (0.15 μCi per reaction) for 10 minutes at 30°C before the addition of Sarkosyl and large excess of unlabeled PC-G. [^{14}C]-PC-G·protein complex was subjected to SDS-polyacrylamide gel electrophoresis, followed by fluorography. a and d, mutant hs-233; b and e, parent PAO 2142; c and f, mutant hs-257.



firmed to have low MICs for beta-lactam antibiotics (Tables 1 and 2). On the other hand, hs-mutants obtained in this experiment possessed almost the same activity (5 to 11×10^{-4} U/mg of protein) of beta-lactamase as that of the parent (Table 2). By the addition of inducer (2 mg/ml of PC-G), beta-lactamase production has been increased up to the level of 1 to 9×10^{-2} U/mg of protein in all the mutants except hs-28. The specific activity of hs-28 remained at the uninduced level, even in the presence of inducer (Table 2). Mutant hs-28 seems to be lacking in inducibility.

[^{14}C]PC-G binding to membrane preparation of mutants, followed by SDS-polyacrylamide gel electrophoresis and fluorography, disclosed that some hs-mutants have some defect in penicillin-binding proteins. In particular, hs-86 and 233 mutant(type I) possessed coincidentally altered electrophoretic mobilities of PBP-1A and 1B, the molecular weight of which was a little higher than those of the parent, as shown in Fig. 1 (data for hs-86 not shown). PBP-1A and 1B may presumably be cooperatively synthesized *in vivo* and these mutants may lack in the enzyme involved in the process of biosynthesis, for example, processing enzyme. Mutants hs-86 and 233 showed partly temperature-sensitive growth even though not selected by temperature. They could form colonies at 40°C, but not at 44°C, whereas the parent could grow even at 44°C. Although we tried to obtain spontaneous revertants of temperature-sensitivity or drug sensitivity to elucidate the relation of defects in PBPs with high sensitivity to a specific group of beta-lactam antibiotics or temperature sensitivity, we have been unsuccessful. These mutants, hs-86 and 233, showed over 10 times higher sensitivity to oxytetracycline, chloramphenicol, mitomycin C and nalidixic acid than did the parent and were sensitive to 100 mcg/ml of gentian violet, although they showed the same level of sensitivity as did the parent to rifampicin, actinomycin D, lysozyme and aminoglycosides such as kanamycin, streptomycin and gentamicin.

Another example of hs-mutants with altered PBP is hs-257. PBP-5 of this mutant moved a very little faster than that of parent on SDS-polyacrylamide gel electrophoresis and lost almost all of its [^{14}C]PC-G binding ability by treatment for 10 minutes at 40°C (Fig. 1, c and f). This PBP-5 lacks activity for releasing [^{14}C]PC-G from [^{14}C]PC-G·protein complex even at 30°C and this mutant also lacks PC-G moderately sensitive D-alanine carboxypeptidase activity, which presumably corresponds to D-alanine carboxypeptidase 1A of *E. coli*[8]. Detailed properties of hs-257 will be reported elsewhere. The growth of mutant hs-257 was inhibited by 100 mcg/ml of actinomycin D and 3.2 mg/ml of lysozyme, at which concentration the growth of the parent was not affected. It showed almost the same sensitivity as the parent to non-beta-lactam antibiotics such as oxytetracycline, chloramphenicol, mitomycin C, nalidixic acid, rifampicin, kanamycin, streptomycin, gentamicin and gentian violet.

The higher sensitivity of hs-mutants for some agents other than beta-lactam antibiotics would suggest that these mutants may have alteration in permeability of drugs through cell envelope, but examination of sensitivity to cell wall-specific phages revealed no difference between parent and hs-mutants obtained in this experiment.

Mutants defective in some PBP could be detected among hs-mutants of *P. aeruginosa*. Mutants hs-233(86) and hs-257 may be useful to elucidate the function of PBP-1A·1B and PBP-5, respectively, in *P. aeruginosa*. The correlation of defects and alteration in PBPs with high sensitivity to beta-lactam antibiotics is now under investigation.

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