ISOLATION OF AN AMINOGLYCOSIDE HYPERSENSITIVE MUTANT AND ITS APPLICATION IN SCREENING

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An aminoglycoside hypersensitive mutant, Kp-126, was isolated from the aminoglycoside-resistant strain, Kp-8, of *Klebsiella pneumoniae* through selection using sorbistin, a non-aminocyclitol-aminoglycoside antibiotic. The mutant Kp-126 was approximately 100-fold more sensitive to sorbistin than the parent strain Kp-8. The mutant also showed hypersensitivity to various aminocyclitol-aminoglycoside antibiotics. *K. pneumoniae* Kp-126 was used in screening and a new aminoglycoside antibiotic, 3,3′-neotrehalosadiamine (BMY-28251), was discovered in the fermentation broths of soil isolate strain of *Bacillus pumilus*.

Aminoglycoside antibiotics constitute a family of therapeutically important anti-infective agents, involving both naturally occurring (streptomycin, kanamycin, gentamicin, sisomicin and tobramycin) and chemically modified antibiotics (amikacin, dibekacin and netilmicin).

Aminoglycoside antibiotics are produced, mainly by actinomycetes but some have been discovered in bacterial fermentation broths. In the course of our screening for aminoglycoside antibiotics, BU-1709 (butirosin)[1], BU-1975 (4′-deoxybutirosin)[2,3] and sorbistin[4,5] were obtained from bacterial cultures and their structures determined.

Although sorbistin has a unique chemical structure and broad antibacterial spectrum, its intrinsic activity is not potent enough to be developed for clinical use. In an effort to discover new types of aminoglycoside antibiotics, we attempted to obtain a sorbistin-hypersensitive mutant which might have specific sensitivity to certain groups of aminoglycoside antibiotics.

It is also anticipated that such mutant would be useful in detecting minor aminoglycoside-type components produced in fermentation broth. Based on this concept, *Klebsiella pneumoniae* type No. 22 (Kp-8), which is a clinical isolate highly resistant to various kinds of antibiotics, was used as the parent strain for mutation studies. A sorbistin-sensitive strain, Kp-126, was isolated from Kp-8 after a series of treatments with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and following selection by the replica plate method. The mutant also showed sensitivity to a wide range of number of the aminocyclitol group of aminoglycoside antibiotics.

The present paper deals with the isolation and characterization of the aminoglycoside hypersensitive mutant Kp-126, and some results of its use in our antibiotic screening.

Meterials and Methods

Bacterial Strains and Isolation of Mutant

K. pneumoniae type 22 No. 3038 (Kp-8) was selected from our culture collection as the parent strain for a mutation study. Induction of mutants was carried out with using NTG according to the method reported by ADELBERG *et al.*[6].

Escherichia coli NIHJ (Ec-1), *K. pneumoniae* D11 (Kp-1) and *Bacillus subtilis* PCI 219 (Bs-1) were used as reference indicator microorganisms.

Media

Heart infusion broth (HIB), Heart infusion agar (HIA) and Mueller-Hinton agar (MHA) were purchased from Difco Laboratories Incorp.[7]. Nutrient broth agar (NBA) containing meat extract 0.5%, peptone 1%, NaCl 0.5% and agar (Eiken) 1.5% was also used. The pH of these media was adjusted to 7.0~7.2 by NaOH before autoclaving.

Characterization of Strains

Physiological and biochemical properties of the strains were examined according to the literature[8] and also by using a diagnostic kit (Enterotube II, Roche) for identification of *Enterobacteriaceae*.

Determination of Virulence

Virulence of the mutant and parent strains was tested in male, albino, ICR-SPF mice weighing 18~20 g. Groups of 10 mice were used for six levels of bacterial challenge. Mice were challenged intravenously with 0.2 ml bacterial suspension ($10^4 \sim 10^{10}$ cells/ml) in physiological saline. The cells were obtained after incubation in HIB for 18 hours at 32°C and the number of viable cells in the saline suspension was determined on NBA plates. The bacterial virulence was assessed by the LD_{50} (cells/mouse) determined 4 days after the challenge.

Determination of Minimum Inhibitory Concentration (MIC)

The MICs of various antibiotics against the mutant and parent strains were determined by the two-fold dilution method on MHA or HIA plates using a Steer's multi-inoculating apparatus. Inoculum was standardized to be a 10^{-4} dilution of bacterial suspention (OD=0.45 at 540 nm) which was prepared from overnight culture in HIB medium.

Paper Disc Assay for Antibiotic Sensitivity

Test strains were incubated in HIB medium at 28°C overnight on a rotary shaker. Each 10-ml molten agar medium (NBA adjusted pH to 9.0) containing 1% inoculum of the test organism was poured into a plastic plate (8.8 cm in diameter). After the agar medium solidified at room temperature, paper discs (8 mm in diameter) containing 35 μl of graded amounts of antibiotics were placed on the agar plates and the plates were incubated at 28°C for 20 hours.

Screening of Antibiotics Active against Kp-126

Strains isolated from soil were cultured in a variety of media with rotary shaking (180 rpm) for 4 days at 28°C. Harvested broths were centrifuged and the supernatants were used for screening. Antibiotic activity against Kp-126 was assayed by the paper disc agar diffusion method.

Results

Isolation of Mutant

Mutation studies using the highly resistant strain Kp-8 were carried out by the procedure as shown in Fig. 1. Kp-8 cells treated with NTG were spread on NBA plates. The plates incubated at 37°C for 2 to 4 days were replicated to the NBA plates containing graded concentrations of sorbistin below the MIC for the parental strain. The colonies which did not grow on the sorbistin-containing plates were picked up and cloned by streaking onto non-medicated NBA plates. After determining the MIC of sorbistin A_1 against the mutants, the most sensitive strain at each step was used as the parent strain for the next step. This selection procedure was repeated 3 times. Among 13 mutants which were inhibited by 3.1 μg/ml of sorbistin A_1, a strain, No. 116, was selected because of its broad sensitivity to various aminoglycoside antibiotics.

The mutant No. 116 was treated with chloramphenicol to induce the resistance to non-amino-

Fig. 1. Isolation procedure of hypersensitive mutant Kp-126.

Kp-8
| NTG (500 μg/ml in 0.05 M Tris-maleate buffer (pH 6.5)) at 37°C for 30 minutes
| replicate on NBA plates containing sorbistin
| (repeat the preceding 2 steps, 3 times)
Sorbistin-sensitive mutants at 3.1 μg/ml
| selected by broad sensitivity to aminoglycoside antibiotics
No. 116
| serial transfer in HIB containing chloramphenicol
| cloned on NBA plated containing chloramphenicol
Chloramphenicol-resistant mutants
| selected by sensitivity to aminoglycoside antibiotics
Kp-126

Table 1. Characteristics of strains Kp-126 and Kp-8.

Test	Response		Method and medium
	Kp-126	Kp-8	
Gram stain	Negative	Negative	HIB
Motility	Negative	Negative	0.3% Agar plate of nutrient broth (Difco)
Oxidase	Negative	Negative	NBA (WURSTER's reagent)
Voges-Proskauer reaction	Weakly positive	Positive	Peptone broth plus 1% glucose
Indole production	Negative	Negative	Peptone broth (KOVACS' reagent)
Methyl red	Weakly positive	Negative	Peptone broth plus 1% glucose
Utilization of citrate	Positive	Positive	SIMMON's citrate medium
H_2S production	Negative	Negative	*
β-Galactosidase	Positive	Positive	Peptone broth plus 1% glucose (*p*-nitrophenyl-β-D-galactopyranoside)
Urease	Weakly positive	Positive	*
Lysine decarboxylase	Positive	Positive	*
Ornitine decarboxylase	Negative	Negative	*
Phenylalanine deaminase	Negative	Negative	*
Gas from glucose	Negative	Positive	*
Utilization of glucose	Positive	Positive	*
Utilization of lactose	Positive	Positive	*
Utilization of arabinose	Positive	Positive	*
Utilization of adonitol	Positive	Positive	*
Utilization of sorbitol	Positive	Positive	*
Utilization of dulcitol	Negative	Negative	*

* Determined by using a diagnostic kit, Enterotube II, Roche.

glycoside antibiotics. After incubation for 2 to 7 days in HIB medium containing graded concentration of chloramphenicol, a full growth culture was transferred into a fresh medium with increasing amounts of the drug. This procedure was repeated three times to obtain strains resistant to 25 μg/ml of chloramphenicol. Among the resistant strains cloned, Kp-126 was finally selected for the present study.

Properties of Mutant Strain Kp-126

The parent strain Kp-8 formed slightly mucoid colonies on the agar medium and possessed prop-

Table 2. Comparative susceptibility of mutant and parent strains to aminoglycoside antibiotics.

Antibiotic	MIC (μg/ml)			
	Kp-126	Kp-8	Kp-1	Ec-1
Sorbistin A_1	<0.2	50	25	>100
Streptomycin	<0.2	>100	3.1	6.3
Neomycin	<0.2	>100	0.8	1.6
Paromomycin	<0.2	>100	0.8	3.1
Kanamycin	<0.2	>100	0.4	3.1
Gentamicin	<0.2	25	0.4	1.6
Butirosin A	<0.2	>100	0.4	1.6
4′-Deoxybutirosin A	<0.2	3.1	0.4	1.6
Amikacin	<0.2	3.1	0.4	1.6
Neamine	<0.2	>100	3.1	12.5

Table 3. Comparative susceptibility of mutant and parent strains to non-aminoglycoside antibiotics.

Antibiotic	MIC (μg/ml)			
	Kp-126	Kp-8	Kp-1	Ec-1
Erythromycin	12.5	50	100	50
Oleandomycin	100	>100	>100	>100
Leucomycin	50	>100	100	100
Cirramycin A_1	25	25	50	12.5
Lincomycin	>100	>100	>100	100
Benzylpenicillin	12.5	>100	50	12.5
Cephalosporin C	1.6	50	12.5	25
Tetracycline	12.5	>100	50	0.8
Chloramphenicol	>100	>100	1.6	1.6
Novobiocin	50	100	6.3	12.5
Colistin	>100	100	0.8	0.2
Aureothricin	100	6.3	3.1	1.6

erties typical of *Klebsiella*; indole and methyl red negative, Voges-Proskauer and citrate positive, non-motile. In addition, this strain was positive for urease, β-galactosidase and lysine decarboxylase, did not produce hydrogen sulfide, and lacked phenylalanine deaminase and ornitine decarboxylase[9]. The physiological and biochemical characteristics of Kp-126 were almost the same as those of the parent strain excepting quantitative differences observed in the formation of gas, acid and acetoin from glucose, and urease activity (Table 1).

The virulence of the mutant to mice ($LD_{50}=3.0\times10^8$ cells/mouse) was several times weaker than that of the parent strain ($LD_{50}=8.9\times10^7$ cells/mouse).

Susceptibility of the Mutant Strain

The susceptibilities of the mutant Kp-126, the parent Kp-8 and the reference indicator strains, Kp-1 and Ec-1, to aminoglycoside antibiotics and non-aminoglycoside antibiotics are comparatively shown in Tables 2 and 3. Kp-8 showed a broad range of resistance to various antibiotics examined except for 4′-deoxybutirosin and amikacin, while Kp-126 was sensitive to all aminoglycoside antibiotics examined below 0.2 μg/ml. MICs of most of aminoglycoside antibiotics to Kp-1 and Ec-1 were in the range of 0.4 μg/ml to 6.3 μg/ml. Thus, the mutant Kp-126 was 4 to 64 times more sensitive to aminoglycoside antibiotics than either Kp-1 and Ec-1, the conventional Gram-negative indicators. However, Kp-126 was less sensitive than Kp-1 and Ec-1 to chloramphenicol, aureothricin and colistin.

Table 4. Paper disc assay of aminoglycoside hypersesitive mutant.

Antibiotic	Concentration (μg/ml)	Zone size (mm)				
		Kp-126	Kp-8	Kp-1	Ec-1	Bs-1
Sorbistin A_1	400	25	12	16	15	16
	100	19	+	+	+	+
	25	13	−	−	−	−
Kanamycin	100	32	−	26	25	27
	25	27	−	21	21	24
	6.3	22	−	14	14	20
Butirosin A	100	33	−	28	25	28
	25	28	−	25	20	25
	6.3	21	−	16	+	16
4′-Deoxybutirosin A	100	34	22	32	27	29
	25	29	16	27	22	25
	6.3	24	+	18	14	17
Xylostatin	100	32	−	28	28	29
	25	27	−	24	24	25
	6.3	21	−	20	17	18
Ribostamycin	100	30	−	27	26	28
	25	26	−	23	23	24
	6.3	20	−	18	14	20
Neamine	100	29	−	26	20	25
	25	25	−	22	16	21
	6.3	18	−	16	12	17
Paromamine	400	20	−	17	18	16
	100	15	−	12	13	12
	25	−	−	−	−	+
Tetracycline	100	13	−	15	12	18
	25	−	−	−	−	14
	6.3	−	−	−	−	+
Chloramphenicol	100	+	−	26	25	22
	25	−	−	18	19	18
	6.3	−	−	−	15	12
Colistin	100	−	−	−	−	−
	25	−	−	−	−	−
	6.3	−	−	−	−	−
Aureothricin	100	13	18	22	19	28
	25	+	14	13	15	22
	6.3	−	−	−	−	13
Cephalosporin C	100	−	−	−	−	18
	25	−	−	−	−	12
	6.3	−	−	−	−	−
Benzylpenicillin	100	−	−	−	20	34
	25	−	−	−	15	31
	6.3	−	−	−	+	28

Paper Disc Agar Diffusion Assay using the Mutant Strain

As shown in Table 4, most of the antibiotics tested gave no inhibitory zone against the parent strain Kp-8, while Kp-126 showed the disc-sensitivity to aminoglycoside antibiotics. In addition, larger inhibitory zones with aminoglycoside antibiotics were observed on plates of Kp-126 than with any other indicator strains.

On the other hand, the sensitivity of Kp-126 to chloramphenicol was as low as that of Kp-8. The disc-sensitivities of all the strains to tetracycline, cephalosporin C and colistin were very low in

comparison with those MIC values.

Thus, the paper disc assay method using Kp-126 was found to be applicable to specific detection of aminoglycoside antibiotics.

Results of Antibiotic Screening with Kp-126

Of about 20,000 soil isolates, ten strains were found to produce antibiotics active against Kp-126. Seven of the screened antibiotics were identified as butirosins[1,10], sorbistins[4], BMY-28160[11], capreomycin[12], and the streptothricin group of antibiotics, respectively. The other two strains produced unidentified antibiotics with uninteresting activity. However, a novel amino sugar antibiotic, BMY-28251[13], was also discovered as specifically active against Kp-126.

Discussion

Antibiotics BMY-28251, discovered through the previously described screen, was specifically active against Kp-126, and was identified as 3,3′-diamino-3,3′-dideoxy-α,β-trehalose[13]. BMY-28251 was also discovered in the fermentation broth of butirosin-producing organism as a previously undetected component in the course of another application of Kp-126 related to studies of butirosin biosynthesis[14].

Mutants of *Pseudomonas aeruginosa*, which showed specific hypersensitivity to aminoglycosides, were genetically studied by MILLS and HOLLOWAY[15] in connection with the alteration of susceptibility to aeruginosin. A similar *Pseudomonas* mutant was also obtained from the highly resistant strain of *P. aeruginosa*, Pa-50, in our laboratory, but its sensitivity to aminoglycoside antibiotics was insufficient to be used for the routine screening.

Our mutant strain Kp-126 is the first example of a *Klebsiella* strain hypersensitive to aminoglycoside antibiotics. Its use in screening is expected to result in the discovery of new aminoglycoside antibiotics.

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