HIGHLY TARGETED SCREENING SYSTEM FOR CARBAPENEM ANTIBIOTICS

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An improved screening system in a search for carbapenem antibiotics from actinomycetes was developed, involving (1) effective isolation of carbapenem antibiotic producers, (2) favorable cultivation as to antibiotic production and (3) simplified identification of the carbapenem antibiotics: (1) A frequency of isolation of carbapenem antibiotic producers from soils was increased by the use of an amoxicillin/clavulanate-containing medium. As many as 20% of the actinomycetes isolated in this medium were found to be carbapenem antibiotic producers, as compared with 0.6% when conventional media were employed. (2) An agar-surface cultivation on oatmeal - yeast extract - malt extract (OMYM) medium, containing oatmeal, yeast extract, malt extract, glucose and trace elements, was most suitable for the antibiotic production. (3) An agar-diffusion assay for identification of carbapenem antibiotics was developed. Although the carbapenem antibiotics are stable against β-lactamases, they were hydrolyzed completely with excess amounts of common β-lactamases. So they could be discriminated from other β-lactam antibiotics and non-β-lactam antibiotics by comparison of the susceptibility profiles for such enzymes.

The carbapenem antibiotics, having potent and broad antibacterial activities and potent β-lactamase inhibitory activities, are unique examples of naturally-occurring β-lactam antibiotics, such as the penam (penicillins), cephem (cephalosporins), 7-methoxycephem (cephamycins), 7-formylaminocephem (cephabacin Fs), monocyclic β-lactam (nocardicins, sulfazecins and SQ-26180), clavam (clavulanic acids) and carbapenem antibiotics.

Several kinds of assay methods have been developed to detect β-lactam antibiotics, such as the growth inhibition assay involving β-lactam antibiotic hypersensitive strains[1,2], the differential growth inhibition assay involving β-lactam antibiotic sensitive- and resistant-strains[3,4], the β-lactamase induction assay[5], the β-lactamase inhibition assay[4,6] and the β-lactamase stability assay[1,2,4]. Utilizing these assay methods in combination, many carbapenem antibiotics have been discovered, such as thienamycins[7], olivanic acids[6,8], epithienamycins[9], the PS series[10,11], the OA-6129 series[12], the C-19393 series[13,14], asparenomycins[15], carpetimycins[16], pluracidomycins[17], the SF-2103 series[18] and SQ 27,860[19].

However, these assay methods are not fully satisfactory for discriminating carbapenem antibiotics from other antibiotics. And there have been few reports as to efficient isolation and cultivation method for antibiotic production.

To detect carbapenem antibiotics from nature more efficiently and more precisely, we have tried to improve the screening system. We have devised an isolation method that increases the frequency of carbapenem antibiotic-producing actinomycetes, a cultivation method for stable and efficient production of the antibiotics, and an assay method for discriminating of carbapenem antibiotics from other antibiotics by use of excess amounts of β-lactamases.

This improved screening system led to the discovery of a new rare actinomycetes, named *Kita-*

satosporia papulosa sp. nov. AB-110[20], that produced a carbapenem antibiotic, AB-110-D[21], which was the *Z* isomer of epithienamycin E[9] (MM 13902[6]) at the C-3 side chain. The antibiotic had never been reported to be actinomycete-product previously.

This paper will describe the development of the new screening system and the results obtained with it.

Materials and Methods

Microorganisms

The known β-lactam antibiotic-producing microorganisms utilized for improvement of our screening system are listed in Table 5.

Comamonas terrigena AJ 2083 was derived as a β-lactam antibiotic sensitive strain from *C. terrigena* ATCC 8461 through mutagenesis.

Media

The media used for the isolation of actinomycetes from soil were as follows: Arginine - vitamin (AV) medium[22], chitin (CH) medium[23] and oatmeal - yeast extract - malt extract (OMYM) medium. The isolation medium contained 20 g/liter of agar, 100 μg/ml of nystatin and 100 μg/ml of cycloheximide. OMYM medium contained of oatmeal 20 g, malt extract 2 g, yeast extract 2 g, glucose 2 g, $CoSO_4 \cdot 7H_2O$ 0.006 g, $ZnSO_4 \cdot 7H_2O$ 0.003 g, $MnSO_4 \cdot 4 \sim 5H_2O$ 0.003 g and $FeSO_4 \cdot 7H_2O$ 0.003 g per liter, adjusted to pH 7.0.

The media used for fermentation were as follows: Tryptone - yeast extract (International Streptomyces Project (ISP)-1) medium, yeast extract - malt extract (ISP-2) medium, oatmeal (ISP-3) medium, inorganic salts - starch (ISP-4) medium, glycerol - asparagine (ISP-5) medium, tyrosine (ISP-7) medium and Czapek's Dox (CD) medium, from Difco Lab.† Bennet's (BE) medium††, egg - albumin (EA) medium[24], SA medium, SQ medium, AV medium[22], CH medium[23] and OMYM medium were prepared in our laboratories. The fermentation media for agar surface culture contained 20 g/liter of agar. SA medium contained of glucose 0.5 g, starch 30 g, soybean meal 30 g, corn steep liquor 5 g, $CaCO_3$ 0.7 g and $CoCl_2 \cdot 6H_2O$ 0.0013 g per liter, adjusted to pH 7.0. SQ medium contained of glucose 25 g, NZ-Amine 4 g, yeast extract 2 g, K_2HPO_4 1 g, Na_2HPO_4 1 g, NH_4Cl 0.5 g and $MgSO_4$ 0.2 g per liter, adjusted to pH 7.0.

The media used for cultivation of the β-lactam antibiotic sensitive strain were nutrient broth (Eiken Co.) and modified nutrient agar (MNA) medium. MNA medium contained meat extract 1.5 g, Polypeptone (Daigo Eiyo Kagaku Co.) 5 g, NaCl 2.5 g and Bacto-agar (Difco Lab.) 10 g per liter, adjusted to pH 7.0.

Antibiotics and β-Lactamases

The antibiotics used were as follows: Benzylpenicillin and streptomycin, purchased from Meiji Seika Kaisha, Ltd., cephalosporin C from Sigma Chemical Company, ceftizoxime from Fujisawa Pharmaceutical Co., Ltd., and amoxicillin/clavulanate (Augmentin) from Beecham Research Laboratories. Cephamycin C was prepared in our laboratories from the broth of *Streptomyces inositovorus* AJ 9408[25]. Clavulanic acid was purified from Augmentin. Imipenem was a gift from Merck & Sharp Dohme Co., nocardicin A from Fujisawa Pharmaceutical Co., Ltd.

The β-lactamases used were as follows: Penicillinase (PCase) from *Bacillus cereus* and cephalosporinase (CPase) from *Enterobacter cloacae* (Sigma Chemical Company; penicillinase type I and type III, respectively). One U was defined as the amount of enzyme which hydrolyzed 1.0 μmol of substrate per minute. The activity of PCase was 2,400 U/mg of protein against benzylpenicillin, and the activity of CPase was 70 U/mg of protein against cephalosporin C.

Kinetic Study on β-Lactam Antibiotic Hydrolysis by β-Lactamases

The β-lactamase-catalyzed hydrolysis reactions of β-lactam antibiotics were studied kinetically

† Difco Manual 10th Ed., Difco Laboratories, Michigan, U.S.A., 1983.

†† ATCC Media Handbook 1st Ed., *Ed.*, R. COTE, ATCC, Maryland, U.S.A., 1984.

by a spectrophotometric method[26,27], measuring the change in UV absorption of the β-lactam ring in 50 mM phosphate buffer (pH 7.0) at 30°C with a Hitachi Model 204 spectrophotometer.

Each β-lactam antibiotic was incubated with enough amount of the enzyme to observe the hydrolysis reaction. From the initial velocity of the reaction, the relative hydrolysis rate (μmol of substrate/mg of protein/minute) was calculated. The wavelengths used for the assay were as follows: 220 nm (clavulanic acid and nocardicin A), 232 nm (benzylpenicillin), 265 nm (cephalosporin C and cephamycin C) and 300 nm (imipenem).

Isolation of Microorganisms from Soil

Actinomycetes for screening use were isolated as follows. Air-dried soil was heated at a temperature between 100 and 120°C for 1 hour and then smashed into a powder[22]. One ml of an appropriately diluted soil suspension in 10 mM phosphate buffer (pH 7.0) was poured, respectively, onto various isolation agar plates with or without selective agents for carbapenem antibiotic producers. Selective agents, such as benzylpenicillin, ceftizoxime and Augmentin, were utilized at the final concentration of 200 μg/ml, respectively. After 3 weeks incubation at 28°C, actinomycetes were transferred onto OMYM agar slants and then cultivated at 28°C for 2 weeks for fermentation.

Cultivation of Microorganisms

The known β-lactam antibiotic producers and isolated actinomycetes were cultivated for antibiotic production on various agar media at 28°C for 3~10 days, or in 10 ml of liquid media in a test tube (23 $\phi \times 195$ mm) on a reciprocal shaker (110 strokes/minute) at 28°C for 2~3 days.

The β-lactam antibiotic-sensitive strain was cultivated in a 500-ml Sakaguchi flask containing 100 ml of nutrient broth on a reciprocal shaker at 30°C for 16 hours, and then aliquots were stored at −85°C for seed cultures.

Agar-diffusion Assay

To establish a discrimination assay for carbapenem antibiotics, the following assay plates were utilized. They consisted of MNA medium (30 ml), the β-lactam antibiotic-sensitive strain (10 μl) and β-lactamase solutions in a wide concentration range (1 ml) in plastic petri dishes (Eiken Co.; 75×225 mm). Paper discs (Toyo Roshi; thick type; 8 mmϕ) loaded with 50 μl of an antibiotic solution were applied on the agar surface of the assay plates. Incubation was carried out at 30°C for 16 hours and then bacterial growth was observed.

To detect carbapenem antibiotics, five kinds of β-lactamase-containing plates were utilized. The final β-lactamase concentrations in each plate were as follows: 0.0001 U/ml of PCase, 0.1 U/ml of PCase, 0.0001 U/ml of CPase, 10 U/ml of CPase and no enzyme, respectively. Paper discs loaded with approximately 40 μl of broth or agar pieces (8 $\phi \times 6$ mm height; approximate volume, 300 μl) taken from the cultivated agar were applied on the agar surface of the assay plates. The growth inhibition patterns due to antibiotics on the five plates were examined to detect carbapenem antibiotics following identification criteria (see Table 3).

Results

Characterization of Carbapenem Antibiotics According to Hydrolysis Kinetics with Excess Amounts of β-Lactamases

Although carbapenem antibiotics such as imipenem are known to be stable to β-lactamases, imipenem was observed to be hydrolyzed completely by excess amounts of β-lactamases. Therefore, the relative rates of hydrolysis of various β-lactam antibiotics, such as benzylpenicillin, cephalosporin C, cephamycin C, nocardicin A, clavulanic acid and imipenem, were investigated on incubation with excess amounts of β-lactamases.

All classes of β-lactam antibiotics could be hydrolyzed, even though extremely large differences existed between the relative hydrolysis rates. Their relative hydrolysis rates (μmol of substrate/mg

of protein/minute) with PCase and CPase are as follows: Benzylpenicillin (2.4×10^{3}; 1.5×10), cephalosporin C (6.7×10; 7.0×10), cephamycin C (1.5×10^{-1}; 5.6), nocardicin A (1.8; 6.1×10^{-3}), clavulanic acid (2.2; 8.0×10^{-3}) and imipenem (2.3×10; 2.0×10^{-1}), as shown in Table 1.

Imipenem exhibited a characteristic susceptibility profile towards PCase and CPase and could be discriminated from the other antibiotics. The others also differ mutually with a single exception that clavulanic acid and nocardicin A exhibited similar profiles.

Table 1. Relative hydrolysis rates of β-lactam antibiotics by β-lactamases.

	PCase	CPase
Benzylpenicillin	2.4×10^{3}	1.5×10
Cephalosporin C	6.7×10	7.0×10
Cephamycin C	1.5×10^{-1}	5.6
Nocardicin A	1.8	6.1×10^{-3}
Clavulanic acid	2.2	8.0×10^{-3}
Imipenem	2.3×10	2.0×10^{-1}

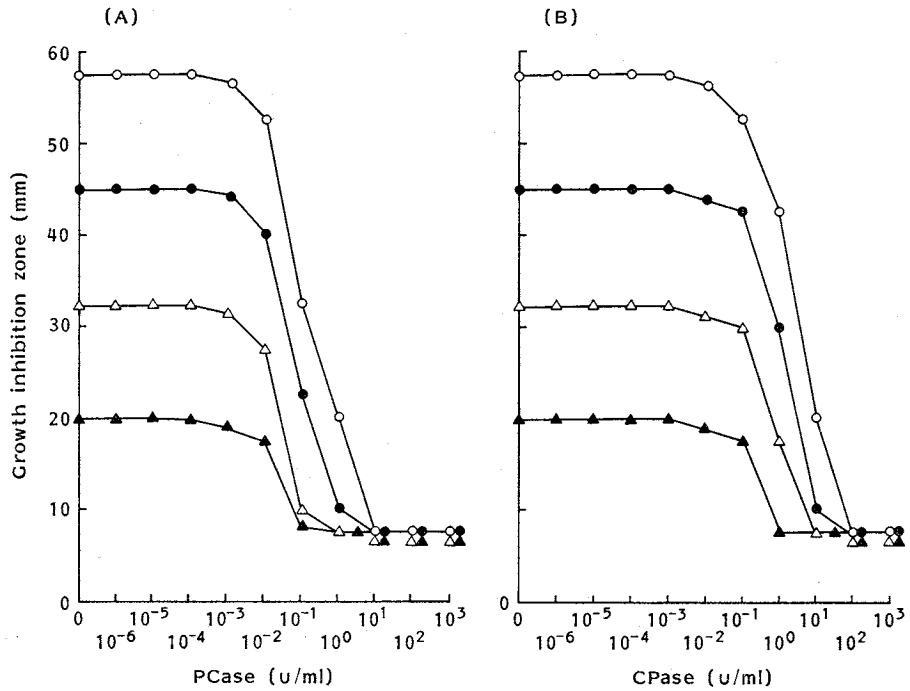
Relative hydrolysis rate of each β-lactam with PCase or CPase (μmol of substrate/mg of protein/minute) was obtained spectrophotometrically from the initial velocity of the hydrolysis reaction by incubating each β-lactam antibiotic with a large amount of β-lactamase.

Agar-diffusion Assay for Carbapenem Antibiotic Discrimination

Utilization of excess amounts of β-lactamases could discriminate imipenem from other antibiotics. Based on this, agar-diffusion assay was devised for a simple and practical discrimination method.

Several kinds of β-lactam antibiotics and a non-β-lactam antibiotic loaded on paper discs were placed on the agar surface of assay plates containing a wide concentration range of β-

Fig. 1. Inactivation of imipenem on plates containing PCase or CPase.
Imipenem ○: 1,000 μg/ml, ● 100 μg/ml, △ 10 μg/ml, ▲ 1 μg/ml.



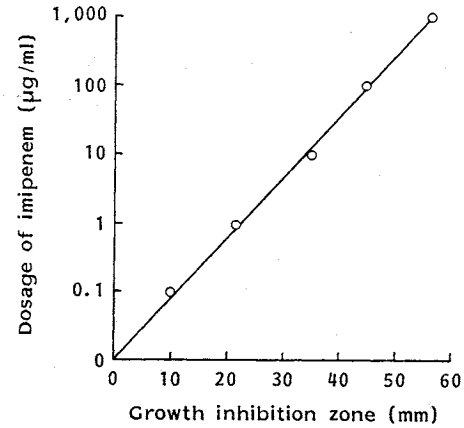
Paper discs impregnated with 50 μl of imipenem solutions in a wide concentration range were placed on *Comamonas terrigena* AJ 2083 plates which contained PCase or CPase solutions in a wide concentration range. After incubation at 30°C for 16 hours, the growth inhibition zones were measured.

lactamases. After incubation, the growth inhibition zones were observed.

The more the antibiotic was inactivated by the enzyme, the smaller the growth inhibition zone became. In the case of from 1 to 1,000 μg/ml of imipenem, the antibiotic was completely inactivated by 10 U/ml of PCase or CPase, as shown in Fig. 1. From the dose-response curve (Fig. 2), the amounts of remaining activity were calculated and plotted against the enzyme dosages, as shown in Fig. 3.

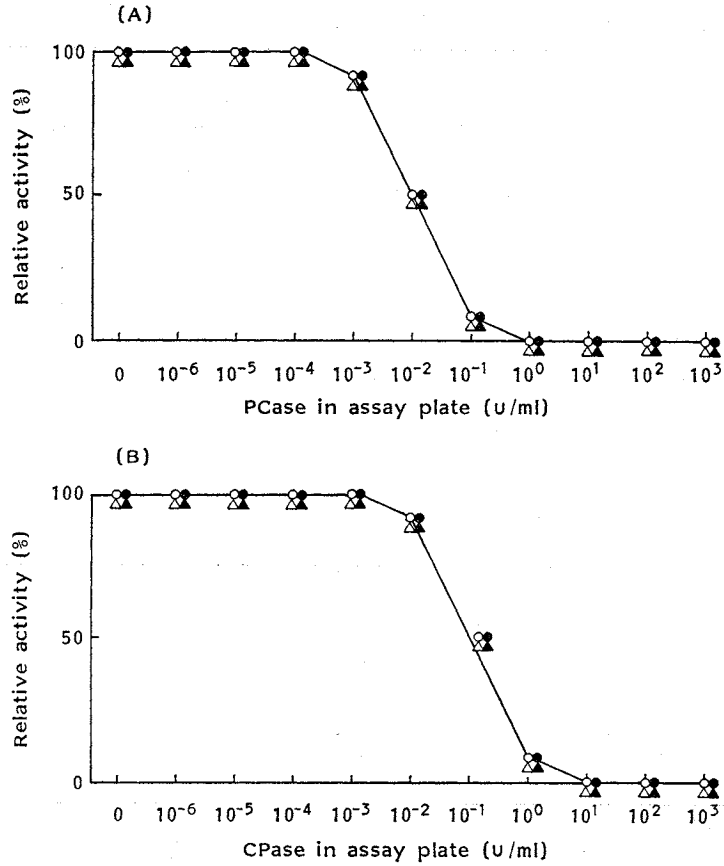
The C_{50} values were defined as the concentrations of the enzymes giving 50% inactivation of an antibiotic, and were obtained from Fig. 3. The C_{50} value for imipenem was 0.01 U/ml of PCase or 0.1 U/ml of CPase.

Fig. 2. Dose-response curve of imipenem on a *Comamonas terrigena* AJ 2083 plate.



Fifty μl of an imipenem solution was loaded on paper discs. Using *C. terrigena* AJ 2083 plate, a dose-response curve was obtained.

Fig. 3. Inactivation of imipenem on plates containing PCase or CPase.
Imipenem ○: 1,000 μg/ml, ● 100 μg/ml, △ 10 μg/ml, ▲ 1 μg/ml.



Amount of remaining activity of imipenem was calculated referring the dose-response curve and then plotted against the enzyme dosages in the assay plates.

In the same way, the C_{50} values for other antibiotics were found to be as follows (U/ml of PCase; U/ml of CPase): Benzylpenicillin (0.00001; 0.00002), cephalosporin C (0.01; 0.00001), cephamycin C (20; 0.0002), nocardicin A (1; 2), clavulanic acid (1; 1) and streptomycin (more than 1,000; more than 1,000). The C_{50} profiles for antibiotics with PCase and CPase are shown in Table 2.

As in the case of Table 1, imipenem showed characteristic profiles as to susceptibility toward PCase and CPase and could be discriminated from other β-lactam antibiotics and a non-β-lactam antibiotic.

The inactivation of various classes of antibiotics by different concentrations of β-lactamases are shown in Table 3. These inactivation patterns were used for the identification criteria of carbapenem antibiotics.

Table 2. C_{50} profiles of β-lactam antibiotics with PCase or CPase.

	PCase (U/ml)	CPase (U/ml)
Benzylpenicillin	0.00001	0.00002
Cephalosporin C	0.01	0.00001
Cephamycin C	20	0.0002
Nocardicin A	1	2
Clavulanic acid	1	1
Imipenem	0.01	0.1
Streptomycin	≧1,000	≧1,000

The concentration of PCase or CPase that gave 50% inactivation of the antibiotic was determined.

Effective Isolation of Carbapenem Antibiotic Producers

During our screening program, some selective agents were evaluated for the effective

Table 3. Simplified identification of carbapenem antibiotics on five kinds of plates.

Antibiotic	Residual activity on MNA medium (%)				
	Without enzyme	With PCase (0.0001 U/ml)	With PCase (0.1 U/ml)	With CPase (0.0001 U/ml)	With CPase (10 U/ml)
Benzylpenicillin	100	0	0	0	0
Cephalosporin C	100	100	0	0	0
Cephamycin C	100	100	100	80	0
Nocardicin A	100	100	100	100	0
Clavulanic acid	100	100	100	100	0
Imipenem	100	100	0	100	0
Streptomycin	100	100	100	100	100

The antimicrobial activities on *Comamonas terrigena* AJ 2083 plates were calculated with reference to the dose-response curve on a *C. terrigena* AJ 2083 plate. Data show relative antimicrobial activities obtained on each plate. The activity on the *C. terrigena* AJ 2083 plate was assumed to be 100%.

Table 4. Effect of selective agents on isolation frequency of carbapenem antibiotic-producing microorganisms and screening performance.

Screening period	Selective agent	Culture isolated	β-Lactam lead	Carbapenem lead	Carbapenem ratio (%)
Period A	None	16,745	108	103	0.6
Period B	None	152	2	1	0.7
	Benzylpenicillin	197	7	6	3.0
	Ceftizoxime	201	5	4	2.0
	Augmentin	124	26	24	19.0
Period C	Augmentin	1,826	376	366	20.0

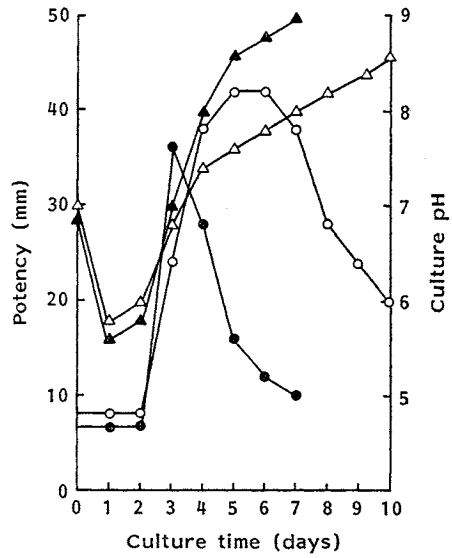
In screening period A, actinomycetes were isolated from many soil samples on conventional media without any selective agent for β-lactam antibiotic producers. In period B, actinomycetes were isolated from same soil samples on four kinds of media without or with selective agents at the final concentration of 200 μg/ml. In period C, actinomycetes were isolated from many soil samples on media with Augmentin.

isolation of carbapenem antibiotic producers. Although these studies were empirical in nature, however, distinct trends were obtained as shown in Table 4.

As few as 0.6% of actinomycetes were carbapenem antibiotic producers in screening period A when they were isolated from many soil samples on the conventional media without any selective agent. This ratio was almost constant even though these samples were applied. In period B, some selective agents at the final concentration of 200 μg/ml in isolation media were evaluated utilizing same soil samples. Augmentin-containing medium was more effective for selective isolation of carbapenem antibiotic producers than non-selective-agent-, benzylpenicillin- or ceftizoxime-containing medium. Therefore, in period C when Augmentin-containing media were utilized for the isolation of actinomycetes from many soil samples, as many as 20% of them were found to be carbapenem antibiotic producers.

Fig. 4. Comparison of carbapenem antibiotic production by *Kitasatosporia papulosa* AB-110 in OMYM agar and submerged cultures.

○ Antimicrobial activity in an agar surface culture, ● antimicrobial activity in submerged culture, △ pH in agar surface culture, ▲ pH in submerged culture.



K. papulosa AB-110 was cultured in OMYM medium by means of agar surface and submerged cultivation. The amount of antibiotic production was expressed as the growth inhibition zone (mm) on *Comamonas terrigena* AJ 2083 plate using the broth from a submerged culture or agar culture. Broth from an agar culture was obtained by squeezing the denatured agar gel after freezing of the gel.

Favorable Cultivation Conditions for Carbapenem Antibiotic Production

In order to search efficiently carbapenem antibiotic producers among isolated microorganisms, cultivation conditions were examined as to effective and stable production of the antibiotics. When an agar surface culture was compared with a shaking liquid culture, higher and more stable excretion of the antibiotic was obtained in the agar surface culture. As an example, Fig. 4 is a comparison between the two cultures of *K. papulosa* sp. nov. AB-110[20], which excreted a carbapenem antibiotic, AB-110-D[21]. Agar-surface cultivation was useful to get higher accumulation and prolonged stability of the activity. Some media, including typical diagnostic test media, were evaluated in agar surface culture using 18 known β-lactam producers. The most suitable medium in an agar surface culture was OMYM medium, as shown in Table 5, which contained oatmeal, yeast extract, malt extract, glucose and trace elements. This cultivation condition was utilized in our practical screening.

Screening Performance

This screening system was applied to the isolation of carbapenem antibiotic-producing actinomycetes. Out of 19,245 strains of actinomycetes screened, 504 carbapenem antibiotic-producing strains were selected, as shown in Table 4. Among the new isolates, we found a rare actinomycete,

Table 5. Evaluation of production media for known β-lactam antibiotic producers.

	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-7	CD	BE	EA	SA	SQ	AV	CH	OMYM
Penicillium chrysogenum ATCC 10135/penicillins	45	45	40	45	42	40	35	45	40	40	42	35	38	45
Cephalosporium polyaleurum ATCC 20359/cephalosporins	50	38	50	32	40	36	40	50	34	40	40	32	50	50
Streptomyces inositovorus AJ 9408/cephamycins	31	37	33	16	17	21	ND	37	30	36	38	ND	15	38
S. olivaceus ATCC 21379/carbapenems	17	40	37	32	20	19	9	36	23	25	ND	14	26	34
S. cremeus ATCC 31358/carbapenems	13	43	41	35	12	29	12	29	30	28	ND	30	32	40
S. lipmanii NRRL 3584/carbapenems	17	36	34	32	20	20	14	37	33	33	36	ND	26	36
S. cattleya NRRL 8057/carbapenems	ND	24	19	24	ND	15	ND	21	13	44	20	ND	15	34
S. flavogriseus NRRL 8139/carbapenems	9	42	38	34	32	28	ND	29	27	26	ND	22	30	40
S. flavus ATCC 3369/carbapenems	ND	30	31	27	19	14	ND	ND	ND	20	ND	ND	22	24
S. argenteolus ATCC 11009/carbapenems	27	28	33	28	20	26	ND	34	28	36	27	18	29	34
S. fulvoviridis ATCC 15863/carbapenems	ND	35	ND	ND	11	15	ND	31	15	24	ND	9	ND	38
S. gedanensis ATCC 4880/carbapenems	13	40	ND	ND	16	37	32	25	33	29	ND	ND	21	42
S. tokunonensis ATCC 31569/carbapenems	34	44	38	38	38	38	26	41	35	38	40	34	32	42
S. sulfonofaciens ATCC 31892/carbapenems	40	46	46	—	40	46	ND	44	—	32	20	—	29	46
S. clavuligerus ATCC 27064/clavulanic acids	35	40	46	34	34	38	—	38	30	40	40	—	30	40
Nocardia uniformis ATCC 21806/monocyclic β-lactams	29	25	30	—	16	21	ND	23	17	21	22	19	15	29
Pseudomonas acidophila IFO 13774/monocyclic β-lactams	ND	ND	13	20	23	19	17	25	—	18	25	19	—	23
Chromobacterium violaceum ATCC 31532/monocyclic β-lactams	ND	ND	ND	ND	ND	ND	ND	13	ND	ND	17	ND	ND	ND

The amounts of β-lactam antibiotics produced are expressed as the diameters of growth inhibition zones (mm) on *Comamonas terrigena* AJ 2083 plates.
ND: Not detected.
—: Not tested.

named *K. papulosa* sp. nov. AB-110[20]. It excreted a carbapenem antibiotic which has not previously been found in nature. The product, AB-110-D[21], was identified as the *Z* isomer of epithienamycin E[9] at the C-3 side chain. The antibiotic was reported in a separate paper[21] and the producer will be also reported in a separate paper[20].

Discussion

Based on the property of β-lactam antibiotics that they lose antibacterial activity on β-lactamase treatment, their detection with β-lactamase-containing plates has been carried out[1,2,4]. However the β-lactamases were used only at ordinary low concentrations, so penicillins and cephalosporins were degraded and detected with those plates. Other antibiotics than non-β-lactam antibiotics were categorized as β-lactamase-stable β-lactam antibiotics, including cephamycins, monocyclic β-lactam antibiotics, clavulanic acids and carbapenem antibiotics. The newly developed assay system, described in this paper, made it easy and precise to discriminate carbapenem antibiotics from other β-lactam antibiotics and non-β-lactam antibiotics by use of excess amounts of β-lactamases.

The supplementation of streptomycin to the culture media was reported to be effective for selecting streptomycin producers from soil[28]. Recently, it was reported that vancomycin was also effective for the selection of glycopeptide producers[29]. However, there have been few reports concerning the efficient isolation of carbapenem antibiotic producers from soil. We found that Augmentin, which is the formulation of amoxicillin and clavulanic acid, increased the isolation frequency of carbapenem antibiotic producers from soil. The main mechanisms of resistance to the β-lactam antibiotics in microorganisms are considered to be as follows: (1) Change in target site (penicillin-binding proteins; PBPs)[30], (2) action of hydrolyzing enzymes (β-lactamases)[31] and (3) reduced access of β-lactam antibiotics to the target sites (permeability barrier)[32]. Almost all carbapenem antibiotic producers isolated in our laboratories were resistant to β-lactam antibiotics, but did not possess β-lactamases (data were not shown). Therefore, it is considered that carbapenem antibiotic producers were resistant to β-lactam antibiotics not because they produced β-lactamases, but because they possessed either a different type of PBPs or a permeability barrier. From these considerations, we employed commercially available Augmentin, a mixture of amoxicillin and clavulanic acid, in the media for the effective isolation of carbapenem antibiotic producers. Clavulanic acid, a strong inhibitor of β-lactamases, did not allow the growth of β-lactamase-containing and β-lactam antibiotic-resistant microorganisms in favor of carbapenem antibiotic producers. Carbapenem antibiotics, if commercially available, could be employed in the isolation medium, bringing about a high isolation frequency of carbapenem antibiotic producers. Amoxicillin alone was considered not to have such effect as in the case of benzylpenicillin or ceftizoxime in Table 4.

We utilized agar surface culture on OMYM medium in our practical screening. Agar surface culture can be usefully employed to obtain higher and stable production of carbapenem antibiotics, without any detailed optimization of many culture conditions. In agar surface culture, the microorganisms grow only on the surface. Nutrients existing at the surface layer are first assimilated by microorganisms and then nutrients in the bottom layers are gradually transferred to the surface. Secondary metabolites were reported to be effectively synthesized when a key nutrient was supplied gradually so that its concentration was kept at a sub-optimum level[33,34]. Also in agar surface culture, the culture-medium is not subjected to aeration, so the partial pressure of carbon dioxide is relatively high. As a result, the increase in culture-pH is considered to be gradual (Fig. 4). A slight change in culture-pH was also found to be favorable for antibiotic accumulation[35,36]. These two factors, a sub-optimum level of nutrients and a slight change in culture-pH, are considered to occur in an agar surface culture, leading to effective and stable production of an antibiotic.

In order to attain the more effective accumulation of carbapenem antibiotics, more precise and detailed optimization study is indispensable including the medium composition and optimum levels of temperature, pH or dissolved oxygen. It is a time- and labor-consuming work. An agar surface culture was useful and important for the practical performance of screening procedure and preliminary characterization of antibiotics.

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