PRODUCTION OF NOVEL ANTIBIOTIC, DOPSISAMINE, BY A NEW SUBSPECIES OF *NOCARDIOPSIS MUTABILIS* WITH MULTIPLE ANTIBIOTIC RESISTANCE

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An actinomycete isolate designated as TS-1980 with multiple resistance to aminoglycoside antibiotics was found to produce novel antibiotics. The strain showed taxonomic features identical to the type strain of *Nocardiopsis mutabilis* except for the temperature range for growth and the utilization of mannitol and raffinose. Based on the capability of growing at lower temperature range, the strain was named *N. mutabilis* subsp. *cryophilis* subsp. nov. Physico-chemical and biological characterization of a purified antibiotic revealed its novel polyamine-type nature with a broad antimicrobial activity. The antibiotic was named dopsisamine.

As reported previously[1,2], naturally-occurring actinomycete strains with multiple resistance to wide ranges of aminoglycoside (AG) antibiotics show a high probability of antibiotic production. Therefore, the isolation and screening of such actinomycete strains were expected to lead to the discovery of novel antibiotics. A soil actinomycete isolate designated as TS-1980, which produced a mixture of multiple antibiotics, was found to meet the above expectation.

In this report, we describe taxonomic characteristics of the strain TS-1980. Although it should be identified as *Nocardiopsis mutabilis*[3], we observed unique morphologies that were not described in the original report on the taxonomy of the organism. In addition, we describe the physico-chemical and biological properties of one of the antibiotics produced by the strain TS-1980 as a polyamine-type antibiotic with a broad antimicrobial activity.

Materials and Methods

Isolation of Organisms

The strain TS-1980 was isolated from a soil collected at Shosenkyo, Yamanashi, Japan. The isolation medium 4PC consisted of the mixture of ISP No. 4 (1 vol) and potato - carrot extract (2 vol), 1.5% agar and 20 μg/ml of two different AG antibiotics (istamycin B and sisomicin). The potato - carrot extract consisted of the filtrate of potato (30 g) and carrot (2.5 g) boiled in 1 liter of water for 30 minutes.

Taxonomical Examination

Morphological, cultural and physiological properties of the strain TS-1980 were examined according to the methods described by Shirling and Gottlieb[4] and Waksman[5]. Detailed observation of mycelial morphologies was performed with the use of a light microscope (XF-Ph-21, Nikon), a transmission electron microscope (EM400, Philips) and a scanning electron microscope (Model S-700, Hitachi) after strain TS-1980 was incubated on the various media at 27°C for 7 to 14 days.

Physiological examinations were carried out in terms of carbon utilization on ISP No. 9 medium containing 1.0% carbon source[4], tolerance to 1 to 10% NaCl on the ISP No. 4 medium, permissive temperature range for growth on the ISP No. 4 medium by the use of a Temperature Gradient Incubator (Toyo Kagaku Sangyo Co., Japan), and susceptibility to 50 μg/ml of 11 different AG antibiotics[1].

Chemical analyses of strain TS-1980 were performed in terms of cell wall type[6], whole cell sugar pattern[7], cellular phospholipids[8], glycolic acid in cell wall[9], menaquinones[10] and nocardiomycolic acid[11].

The type cultures used for taxonomic comparison were obtained from IFO (Institute for Fermentation, Osaka).

Fermentation

The medium used for the production of the antibiotics consisted of dextrin 2.0%, glucose 0.2%, soybean meal 2.0%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.3% and K_2HPO_4 0.1%. A slant culture of the strain TS-1980 on ISP No. 4 medium was inoculated into 100 ml of the medium in a 500-ml vol of Erlenmeyer flask and incubated at 27°C for 96 hours on a rotary shaker (180 rpm). One ml of this culture was then transferred to 100 ml of the fresh medium and incubated for 120 hours in the same way as above. The antibiotic activity of the culture was assayed with *Bacillus subtilis* PCI 219 as a test organism.

Purification of an Active Substance

The culture broth (total 15 liters) was filtered at pH 2.0 (adjusted with conc HCl) and pH 7.0 (adjusted with 6 N NaOH), successively, by using Hyflo Super-Cel (Johns-Manville Co., USA) as a filter aid. The antibiotic principle in the filtrate (14 liters) was adsorbed on a column of Amberlite IRC-50 (70% Na^+, 500 ml). After the column was washed with water (5 liters), 1 N HCl (2 liters) and water (5 liters), successively, the antibiotics were eluted with 0.05 N HCl - 80% aq MeOH (2 liters). Active fractions were pooled, neutralized with Amberlite IRA-45 (OH^-), evaporated to 10 ml *in vacuo* and lyophilized.

The resultant crude yellow powder (1.89 g) was dissolved in 10 ml of 0.3 M NaCl solution and charged on a column of CM-Sephadex C-25 which was pre-equilibrated with 0.3 M NaCl (500 ml). Elution with 5 liters of NaCl solution with a concentration gradient of 0.3 M to 1.0 M at a flow rate of 100 ml/hour provided roughly three peaks of active fractions (Fig. 1). The active fractions of the peak designated as III were combined and loaded on a column of Amberlite XAD-2 (50 ml). The column was thoroughly washed with deionized water (1 liter) and the antibiotics were eluted with 0.05 N HCl - 50% aq acetone. The eluate was neutralized with Amberlite IRA-45 (OH^-) and evaporated to 5 ml *in vacuo* and lyophilized.

A pale yellow powder of the antibiotics thus obtained (258 mg) was then charged on a column (2 cm × 100 cm) of silica gel (Wakogel C-200) and developed with $CHCl_3$ - MeOH - NH_4OH (2: 2: 1). The fractionated eluate was monitored with antibiotic activity and silica gel TLC using $CHCl_3$ - MeOH - AcOH - H_2O (3: 1: 1: 0.2) as a developing solvent. Fractions showing a blue color spot with anisaldehyde sulfate (Rf 0.32) were pooled and concentrated to dryness. The residue was dissolved in 1 ml of deionized water and passed through a column of Dowex 1-X2 (OH^-). After adjusting the pH to 5.0 with 1 N H_2SO_4, the effluent was passed through a column of Dowex 1-X2 (SO_4^{2-}). The eluate was evaporated to dryness and dissolved in 0.5 ml of 50% aq MeOH. This aq MeOH solution containing the antibiotics was passed through a column of Sephadex LH-20 (2 cm × 100 cm) equilibrated with 50% aq MeOH and developed with 50% aq MeOH. Each fraction (2 ml) was monitored by TLC. The fractions positive to anisaldehyde color reaction were combined and concentrated to 2 ml and lyophilized. Thus, 91.8 mg of white powder of a pure antibiotic exhibiting a single spot by the color reaction with anisaldehyde sulfate on a silica gel TLC plate was obtained.

Determination of Antimicrobial Activity

To determine the minimal inhibitory concentration of the purified antibiotic, bacteria were incubated at 37°C for 18 hours in Mueller-Hinton agar (Difco), and yeasts and molds were incubated at 27°C for 18 hours in nutrient agar (Difco) supplemented with 1% glucose.

Table 1. Taxonomic features of strain TS-1980.

Cultural characteristics*		
Growth	Good ~ abundant:	W2, ISP No. 2, 4, 5 & 7, NA
	Moderate:	W1, ISP No. 3
Reverse side color	Pale yellow:	W1 & 2, ISP No. 2, 3, 4, 5, 6 & 7, NA
Aerial mass color	White:	W1, ISP No. 2, 5 & 6, NA
	White ~ pale yellow:	W2, ISP No. 3, 4 & 7
Morphological characteristics		
Substrate mycelium	"Zig-zag" hyphae: W1, ISP No. 6, NA	
Aerial mycerium	Long straight hyphae (ϕ 0.2 ~ 0.5 μm)	
	Electron dense particles	
	Segmentation	
	Sporangium-like body	
	Sclerotium-like body	
Chemical characteristics		
Cell wall type	IIIC (*meso*- and LL-DAP, galactose)	
Acyl type	Acetyl	
Phospholipid	PIV	
Menaquinone	MK9 (H4)	
Nocardiomycolic acid	Negative	
Staining	Gram-positive, non-acid fast, positive to nucleus	
Physiological characteristics		
Permissive temperature	8 ~ 33°C (optimal range 21 ~ 27°C)	
Carbon utilization Positive:	Glucose, sucrose, xylose, inositol, mannitol, fructose, raffinose	
Negative:	Rhamnose, arabinose (variable)	
Melanoid pigment	Negative	
Milk coagulation & peptonization	Negative	
Nitrate reduction	Positive	
Gelatin liquefaction	Positive	
NaCl tolerance	1 ~ 3%	

* W1; Sucrose - nitrate agar (Waksman No. 1 medium), W2; glucose - asparagine agar (Waksman No. 2 medium), ISP No. 2; yeast - malt extract agar, ISP No. 3; oatmeal agar, ISP No. 4; inorganic salts starch agar, ISP No. 5; glycerol - asparagine agar, ISP No. 6; peptone - yeast extract - iron agar, ISP No. 7; tyrosine agar, NA; nutrient agar.

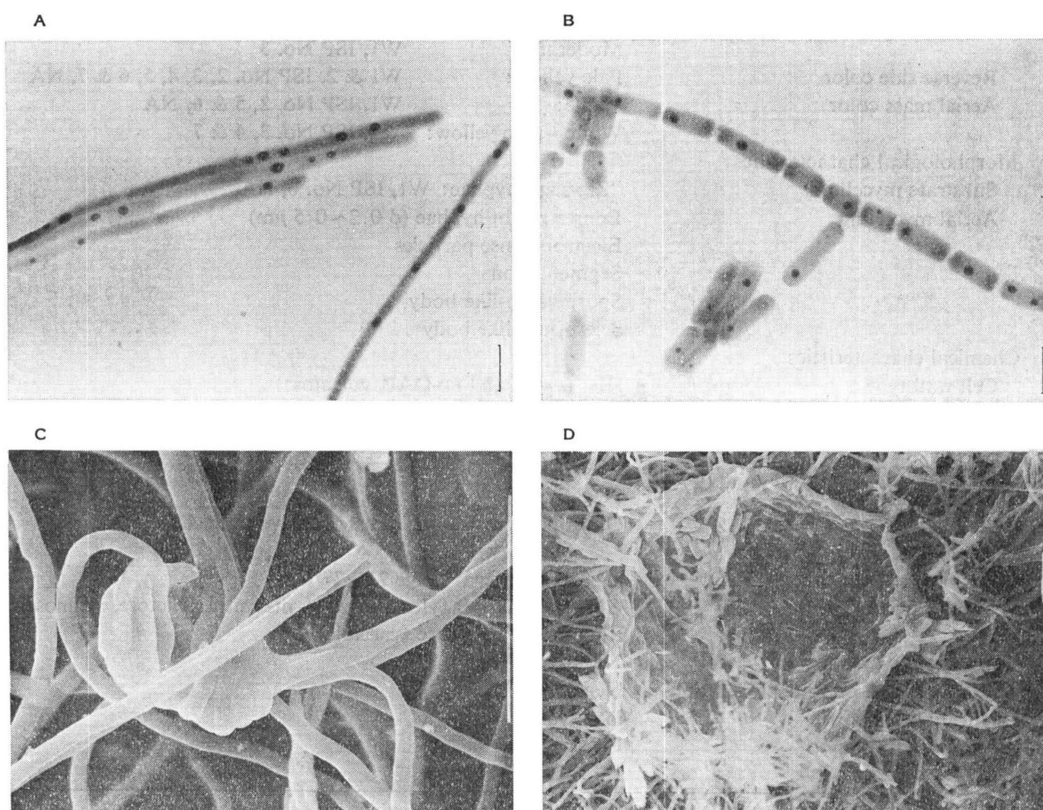
Results

Taxonomic Features of the Strain TS-1980

Taxonomic features of the strain TS-1980 were summarized in Table 1. The strain showed good or abundant growth with white to pale yellow aerial mass color on various media. Aerial mycelium consisting of long straight and thin hyphae formed a variety of unique morphological structures. As shown in Photo 1, they divided into long segments (Photo 1-A) which in turn subdivided into short rods with smooth surface (Photo 1-B). Highly electron dense round particles were observed in each segment (Photo 1-A). Hyphae were so adhesive that they developed a bundle or tight loops, resulting in the formation of sporangium- or sclerotium-like structures (Photos 1-C and -D). The substrate mycelium was observed to be "nocardioid"-like zig-zag hyphae. The mycelium grown on agar media was Gram-positive but not acid fast. Nucleus staining revealed the presence of positive particles.

Chemical analyses of cell wall and whole cell sugar revealed other unique aspects of the strain TS-1980. *Meso*-diaminopimelic acid (DAP) was detected together with lesser amount of LL-DAP in the acid hydrolysate of the purified cell wall preparation. Alanine, glutamic acid and galactose were also detected, but glycine and arabinose were not. Whole cell acid hydrolysate contained galactose,

Photo 1. Morphology of strain TS-1980.
A, C & D: ISP No. 4 medium, B: W1 medium, A & B: a mark equals 1 μm, C & D: a mark equals 5 μm.

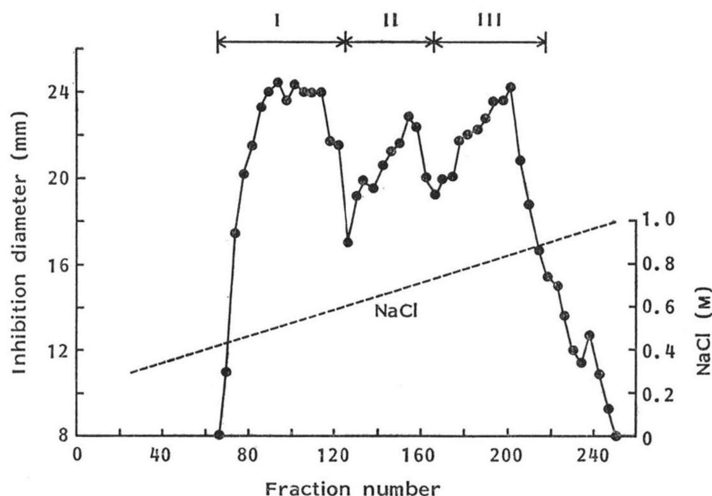


glucose, rhamnose and ribose, but not arabinose, madurose and xylose. These chemical composition of the strain TS-1980 would not allow assignment to any one of the known cell wall types or the known whole cell sugar patterns. However, it would seem to be closely related to type III cell wall and type C whole cell sugar pattern as in the case of *Actinosynnema*[12]. The types of acyl group of cell wall, phospholipids and menaquinone were acetyl, PIV and MK9 (H4), respectively. Nocardiomycolic acid was not detected.

The strain TS-1980 grew at temperature range of 8~33°C with the optimum range of 21~27°C. In addition, the strain was resistant to 50 μg/ml of the following 11 AG antibiotics; streptomycin, kanamycin A, dibekacin, gentamicin C, ribostamycin, istamycin B, paromomycin, lividomycin A, butirosin A, neomycin B and neamine.

Based on the above features, *Actinoplanacae* and nocardioform actinomycetes[13] were chosen as candidate taxa for the strain TS-1980. The comparative examination of type strains belonging to the species of these two taxa together with the strain TS-1980 revealed that *N. mutabilis* IFO 14310 (ATCC 31520) had the taxonomic features identical to those of the strain TS-1980 with exception of some physiological difference. The type strain (IFO 14310) of *N. mutabilis* grew at the range of 15~42°C, whereas the strain TS-1980 at 8~33°C. The type strain did not utilize mannitol and raffinose as sole carbon sources and was sensitive to kanamycin A, while the strain TS-1980 gave the opposite results in these respects. Thus, the most significant and distinct difference lay in the permissive tem-

Fig. 1. CM-Sephadex C-25 column chromatography.



perature for growth. The strain TS-1980 was therefore classified as a new subspecies of *N. mutabilis*. *N. mutabilis* subsp. *cryophilis* subsp. nov. Takahashi, Hotta et Okami was the designation given to the strain TS-1980 which was nominated as the type and deposited at IFO. It should be noted that *N. mutabilis* IFO 14310 exhibited the same unique morphological structures as those observed in the strain TS-1980, because these morphologies were not described in the original report on the type strain[3].

Physico-chemical Properties of the Antibiotic

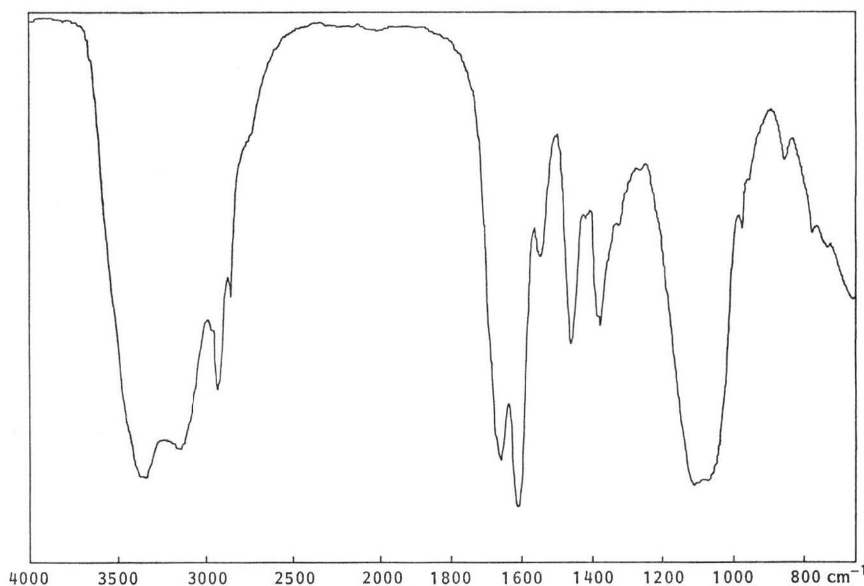
The strain TS-1980 produced a mixture of antibiotic substances with similar physico-chemical properties. As shown in Fig. 1, at least three peaks appeared in the antibiotic activity curve after CM-Sephadex C-25 chromatography. In the course of the purification, each peak was found to contain several antibiotic substances (data not shown). Thus, at least 20 antibiotics were contained in the filtrate of the culture broth. One of the antibiotics in the peak designated as III in Fig. 1 was purified as described in Materials and Methods, and named dopsisamine.

Physico-chemical properties of dopsisamine were summarized in Table 2. The antibiotic is a hygroscopic white powder which is water soluble and basic, and positive to Sakaguchi and anisaldehyde sulfate color reactions. The IR spectrum was shown in Fig. 2. The UV absorption spectrum showed two major peaks at 206 and 305 nm in phosphate buffer (pH 6.8). In 0.1 N HCl, the peak at 305 nm shifted to 322 nm. Elemental analysis and the spectra of FD-MS, SI-MS, ^{1}H NMR and ^{13}C NMR established the molecular formula of $C_{30}H_{55}N_{11}O_3 \cdot 3/2H_2SO_4 \cdot H_2O$. The ^{1}H NMR (in D_2O, 400 MHz) spectrum (Fig. 3) indicated the presence of one olefinic proton (5.16 ppm), two methyl groups (1.67 and 1.71 ppm) and one methoxy group (3.95 ppm). Furthermore, decoupling analysis indicated the possible presence of two kinds of carbon chain linked to the nitrogen atom, $2\times(NCH_2CH_2CH_2N)$ and $2\times(NCH_2CH_2CH_2C)$, and tri-substituted olefin, $(CH_3)_2C{=}C\langle^{H}_{CH_2-}$. The reaction of dopsisamine with acetylacetone in water in the presence of potassium carbonate gave bis(dimethylpyrimidyl) derivative (*m/z* 745 and 746 in EI-MS and SI-MS, respectively), indicating the probable existence of guanidino carbons which could correspond to two out of three signals at 157.9, 157.4 and 157.1 ppm in the chemical shift data of the ^{13}C NMR spectrum (Table 3). These physico-chemical properties

Table 2. Physico-chemical properties of dopsisamine sulfate.

Appearance	White powder
Melting point	155~160°C
UV max ($E^{1\%}_{1cm}$) nm	
Phosphate buffer (pH 6.8)	206 (183), 220 (sh, 142), 280 (sh, 53), 305 (126)
0.1 N HCl	206 (183), 235 (149), 322 (168)
0.1 N NaOH	216 (212), 280 (sh, 53), 306 (129)
Molecular weight FD-MS $(M+H)^+$	618
SI-MS $(M+Na)^+$	640
Molecular formula	$C_{30}H_{55}N_{11}O_3 \cdot 3/2H_2SO_4 \cdot H_2O$
Elemental analysis Found:	C 46.22, H 8.38, N 19.07, S 6.09
Calcd:	C 46.04, H 7.67, N 19.69, S 6.14
Solubility Soluble:	H_2O
Insoluble:	MeOH (emulsion), EtOH (emulsion), BuOH, acetone, EtOAc, $CHCl_3$, benzene
Color reaction Positive:	Sakaguchi, anisaldehyde - H_2SO_4
Negative:	Ninhydrin, aniline phthalate
Rf values on silica gel TLC	0.32 ($CHCl_3$ - MeOH - AcOH - H_2O, 3:1:1:0.2)
	0.46 (BuOH - AcOH - H_2O, 3:1:1)
High voltage paper electrophoresis (3,000 Volt)	Rm 1.34 (Ala 1.00)

Fig. 2. IR spectrum of dopsisamine sulfate (KBr).



indicate that dopsisamine is a new polyamine-type antibiotic different from any known antibiotic ever reported.

Biological Activities of Dopsisamine

Table 4 shows MIC of dopsisamine against bacteria, yeasts and molds. It was active to Gram-positive and Gram-negative bacteria except *Serratia* and *Pseudomonas*. A high activity was also observed in *Saccharomyces cerevisiae* and weak activity to *Xanthomonas oryzae*, *Candida albicans* and molds. Acute toxicity (LD_{50}, iv) in mice was about 0.6 mg/kg.

Fig. 3. ^{1}H NMR spectrum of dopsisamine sulfate (400 MHz, D_2O).

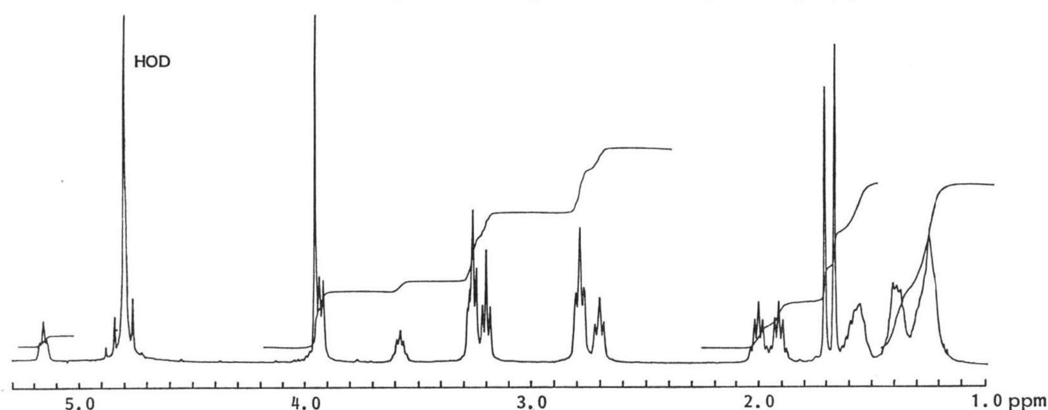


Table 3. Chemical shifts of carbon in the ^{13}C NMR (100.4 MHz, D_2O) spectrum of dopsisamine.

Carbon number	Chemical shift (δ)	Multiplicity*
1	157.9	s
2	157.4	s
3	157.1	s
4	149.3	s
5	146.7	s
6	143.6	s
7	139.9	s
8	118.1	d
9	72.1	d
10	54.8	q
11	49.3	t
12	47.8	t
13	41.7	t
14	41.6	t
15	37.0	t
16	36.5	t
17	33.2	t
18	30.6	t
19	30.2	t
20	29.7	t
21	29.6	t
22	29.3	t
23	29.2	t
24	28.1	t
25	27.7	t
26	27.5	t
27	25.9	q
28	25.6	t
29	23.0	t
30	18.2	q

* q=Quartet, t=triplet, d=doublet, s=singlet.

Table 4. Antimicrobial activity of dopsisamine.

Organisms	MIC (μg/ml)
Staphylococcus aureus 209P	0.39
Micrococcus luteus IFO 3333	0.78
Bacillus subtilis PCI 219	6.25
Escherichia coli NIHJ	3.12
Proteus vulgaris OX19	6.25
Serratia marcescens	100
Klebsiella pneumoniae PCI 602	6.25
Mycobacterium smegmatis ATCC 607	3.12*
Pseudomonas aeruginosa No. 12	100
Candida albicans 3147	25
Saccharomyces cerevisiae F-7	3.12
Pyricularia oryzae P-2	50
Xanthomonas oryzae	25
Aspergillus niger F-16	50
Trichophyton mentagrophytes (833)	50

* 37°C, 40 hours.

Discussion

Our first step of screening program for new antibiotics has been directed to select actinomycete isolates or clones with multiple resistance to AG antibiotics and/or unique AG antibiotics resistance pattern. This strategy was established on the basis of our findings[1,2] that actinomycete strains with multiple AG antibiotic resistance exhibit very high probability of antibiotic production, and that unique resistance patterns correlate strongly with the types of antibiotics produced. This approach resulted in the discovery of indolizomycin by selecting a clone with a unique multiple AG antibiotic resistance pattern after a fusion treatment between *Streptomyces* strains belonging to two different species[14~16]. In the present paper, a new polyamine-type antibiotic was discovered by selecting a novel actinomycete

isolate with multiple AG antibiotic resistance. Although the strain TS-1980 was identified as *N. mutabilis,* the antibiotic produced was different from polynitroxin[17] produced by the type strain of *N. mutabilis.* The type strain is sensitive to kanamycin A to which the strain TS-1980 is resistant, although both strains are resistant to the other 10 AG antibiotics tested. Therefore, our hypothesis that a strain-specific resistance-pattern may represent strain specificity of antibiotic synthesis was again supported. Recently, resistance genes and the biosynthetic genes for the same antibiotics were reported to be located in the same cluster[18,19]. Therefore, our approach might be rationalized for the discovery of new antibiotics.

The strain TS-1980 exhibited taxonomic features identical with those of *N. mutabilis* IFO 14310. However, we observed distinct morphological features which were not described in speciation of *N. mutabilis* Shearer[3]. The sclerotium- or sporangium-like bodies were not described as major morphological characteristics of *Nocardiopsis.* Whole cell of *N. mutabilis* including the strain TS-1980 contained menaquinone MK9 (H4), phospholipid PIV and galactose, while that of type species of *Nocardiopsis, N. dassonvillei* contained MK10, phospholipid PIII and no galactose. In addition, *N. mutabilis* markedly differs from the other species of *Nocardiopsis* in respect to resistance to AG antibiotics. All type strains of species in *Nocardiopsis* except *N. mutabilis* were sensitive to 11 AG antibiotics tested, while *N. mutabilis* IFO 14310 and TS-1980 were resistant to 10 and 11 AG antibiotics, respectively. These facts indicate that *N. mutabilis* might need an appropriate taxonomic niche different from the other species of *Nocardiopsis.*

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