A SELECTIVE ISOLATION PROCEDURE FOR *MICROMONOSPORA*

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A selective medium containing 25 to 50 μg per ml of tunicamycin was devised to isolate micromonosporae from soil samples, making possible simple, preferential isolation of a variety of *Micromonospora*. When a large amount of Gram-negative bacteria was present in a sample, alkaline treatment (0.01 N NaOH, 5~10 minutes at 15°C) was employed to reduce the numbers. Using the tunicamycin agar medium, 1,585 strains of presumably different micromonosporae were obtained from 400 soil samples collected from various regions around the world. In average, 4 different *Micromonospora* strains could be isolated from one soil sample. This tunicamycin method made possible a concentrated screening method for new antibiotics from *Micromonospora*.

Micromonosporaceae bacteria have been recognized as one of the most important sources of new antibiotics. Many of the aminoglycoside type (gentamicins,[1] sisomicin,[2] G-418,[3] verdamicin,[4] sagamicin,[5] and G-52[6]), the macrolide type (megalomicins,[7] rosamicin,[8] KX-41-B_2,[9] juvenimicin,[13] and M-4365[10]), and miscellaneous types (iodinin,[11] tetrenolin,[12] and bottromycins[14]) have been found in strains of *Micromonospora*.

SOLOVIEVA and SINGAL[15] reported that 1) representative *Micromonospora* strains are distributed widely in soils of various geographical regions and 2) they occur preferably in moist soils. SINGAL *et al.*[16] showed that a) *Micromonospora* strains predominated in moist soils and slits and b) their content with respect to all isolated *Streptomycetaceae* amounts to 67 to 83% in moist soils and silts but only 6 to 11% in ordinary soil samples.

Selective isolation of this group of bacteria was undertaken in 1957 by NONOMURA and OHARA,[17] who isolated *Microbispora* strains from soils by spreading the soil particles over soil-extract agar plates. They[18] also devised a procedure for preferential isolation of *Microbispora* and *Streptosporangium* by heating soil samples at 120°C for 1 hour and then plating them on a chemically defined medium (AV-medium) containing nystatin and cycloheximide. IVANITSUKAYA, *et al.*[19] used gentamicins to isolate a group of *Micromonospora* from soil samples.

The present study was initiated to devise a more efficient isolation method for *Micromonospora*. We preliminarily examined about 35 kinds of antibiotics for their sensitivities to various Gram-positive and -negative bacteria including micromonosporae. The tests showed that tunicamycin[20] inhibited growth of all Gram-positive bacteria except *Micromonospora*. This insensitivity of *Micromonospora* strains to tunicamycin was used as the basis of our method.

Materials and Methods

Bacteria Used

Sixty-three strains of Gram-positive and -negative bacteria including *Micromonospora* (see Table

1) were used in sensitivity tests against tunicamycin. Most of the strains were obtained from the Institute of Fermentation, Osaka (IFO), the Northern Regional Research Laboratories, U.S.A. (NRRL), and the American Type Culture Collection (ATCC). The others were isolated and characterized in this laboratory.

Sensitivity Test to Tunicamycin

An agar dilution method was used to determine the sensitivities of various Gram-positive and -negative bacteria against tunicamycin. About one μl of 10^6 to 10^7 CFU/ml cell suspension of each test microorganism was inoculated onto Gly-IM agar medium containing 10 and 50 μg/ml of tunicamycin. Gly-IM agar: 0.5% glycerol, 0.25% Polypeptone (Daigo Eiyo Co., Ltd., Osaka), 0.25% beef extract (Kyokuto Co., Ltd., Tokyo), 0.25% yeast extract (Difco), 0.25% Bacto Soytone (Difco), 0.3% NaCl and 1.25% agar. The inoculated plates were incubated at 28°C for 1 to 14 days, and the growth of each inoculate at 2, 4, and 6 days (Gram-positive and -negative bacteria) or 3, 7, 10 and 14 days (*Streptomycetaceae* bacteria) were measured by naked eye.

Survival Test in 0.01 N NaOH Solution

The survival tests of various Gram-positive and -negative bacteria in 0.01 N NaOH solution were performed according to following process: One ml of each test organism suspension ($10^3 \sim 10^6$ CFU/ml in sterile saline containing 0.01% $MgSO_4 \cdot 7H_2O$, stationary phase) was combined with 9 ml of 0.01 N NaOH solution (sterile) and incubated at about 15°C for 5 to 30 minutes. At 5, 10 and 30 minutes incubation, one ml of the mixture was taken out, neutralized with 0.1 N HCl (sterile) to pH 6 to 7 under cooling, and adjusted to a volume of 2.0 ml with sterile saline. One ml portion of the neutralized sample was then plated on Gly-IM agar and incubated at 28°C or 37°C for 1 to 7 days. The surviving colonies were counted from the incubated plates and the survival ratio at 5, 10 or 30 minutes treatment was calculated as follows; [(viable cells/ml at a treatment interval) − (viable cells/ml at starting stage)] × 100%.

Selective Isolation Procedure for *Micromonospora*

About 0.5 g of fresh soil sample was suspended in 5 ml of sterile saline containing 0.01% $MgSO_4 \cdot 7H_2O$ and 0.002% Tween 20, and stirred vigorously with two or three glass beads (about 4 mm in diameter) by a Micro Thermo-Mixer (Model TM-101, Thermonics Co., Ltd., Tokyo) for 1 minute at room temperature (about 15°C). Next, the suspension was placed in a vacuum desiccator for about 30 minutes to eliminate air from the mixture. The vacuum-treated sample was again stirred by the Thermo-Mixer for about 30 seconds, and diluted to one-tenth concentration with the sterile saline. One milliliter of the diluent was combined with 9 ml of 0.01 N NaOH and the mixture was left standing for 5 to 10 minutes at about 15°C. The mixture was then neutralized with 0.1 N HCl to pH 6 to 7 under cooling. The neutralized mixture was plated with serial dilution on the isolation medium containing 25 μg per ml of tunicamycin. Modified Bennet's medium (1% glucose, 0.2% Casamino acids, 0.2% yeast extract, 0.1% beef extract, 1.5% agar, pH 7.0) was used in this procedure. The inoculated plates were incubated at 28°C for 14 to 21 days. Moisture chamber was preferable. If necessary, 30 μg per ml of cycloheximide was added to the isolation medium.

Results

Sensitivity of Tunicamycin to Various Gram-positive and -negative Bacteria

Sixty-one strains of various Gram-positive and -negative bacteria including micromonosporae were tested for their sensitivities against tunicamycin. The micro agar-dilution techinique was used with the results shown in Table 1.

All the Gram-positive bacteria tested were susceptible to this antibiotic except *Micromonospora* strains. Namely, seven strains of asporogenous Gram-positive aerobes, 13 strains of *Bacillus,* and 15 strains of *Streptomyces* bacteria were all sensitive to 10 and 50 μg per ml of tunicamycin. The nine strains of *Micromonospora* tested were however all insensitive to this antibiotic, with the exception of *M. echinospora* NRRL 2985 which gave weak growth at 10 μg/ml and no growth at 50 μg/ml, and with

Table 1. Susceptibility test of Gram-positive and -negative bacteria to tunicamycin[a].

	Tunicamycin								
	0 μg/ml			10 μg/ml			50 μg/ml		
Days	3	7	10	3	7	10	3	7	10
A. *Streptomyces*:									
1. *S. griseus* NRRL 3851	3	3	3	0	0	0	0	0	0
2. *S. lactamdurans* NRRL 3802	2	3	3	0	0	±	0	0	0
3. *S. lincolnensis* ATCC 25466	2	3	3	0	0	0	0	0	0
4. *S. kanamyceticus* ATCC 12853	3	3	3	0	0	0	0	0	0
5. *S. tenebrarius* ATCC 17920	2	3	3	0	0	0	0	0	0
6. *S. vinaceus* ATCC 25510	3	3	3	0	0	0	0	0	0
7. *S. humidus* ATCC 12760	3	3	3	0	0	0	0	0	0
8. *S. fradiae* Waksman 3535	2	3	3	±	2	2	0	1	2
9. *S. ribosidificus* ATCC 21294	2	3	3	0	0	0	0	0	0
10. *S. kasugaensis* ATCC 15714	2	3	3	0	0	0	0	0	0
11. *S. levoris* ATCC 5876	3	3	3	0	0	0	0	0	0
12. *S. erythraeus* F-188	2	3	3	0	0	0	0	0	0
13. *S. hygroscopicus* K-775	3	3	3	0	0	0	0	0	0
14. *S. antibioticus* E-662	2	3	3	0	0	0	0	0	0
15. *S. lavendulae* K-433	2	3	3	0	0	0	0	0	0
B. *Micromonospora*:									
1. *M. purpurea* NRRL 2953		2	3		2	2		2	2
2. *M. halophytica* subsp. *nigra* NRRL 3097	3	3	3	3	3	3	2	3	3
3. *M. echinospora* NRRL 2985	2	3	3	±	1	2	0	0	0
4. *M. carbonacea* NRRL 2972	3		3	3	3	3	3	3	3
5. *M. chalcea* subsp. *flavida* NRRL 3222	3	3	3	3	3	3	3	3	3
6. *M. megalomicea* NRRL 3274	3	3	3	0	0	3	0	0	1
7. *M. echinospora* subsp. *inyoensis* NRRL 3292		3	3		3	3		3	3
8. *M. rosaria* NRRL 3718	±	3	3	±	3	3	0	3	3
9. *M. grisea* NRRL 3800	3	3	3	2	3	3	0	1	1

	Tunicamycin					
	0 μg/ml		10 μg/ml		50 μg/ml	
Days	2	6	2	6	2	6
C. Asporogenous Gram-positive aerobes:						
1. *Brevibacterium ammoniagenes* ATCC 6871	3	3	0	0	0	0
2. *Brevibacterium linens* ATCC 9172	2	3	0	0	0	0
3. *Corynebacterium glutamicum* ATCC 13058	3	3	0	0	0	0
4. *Micrococcus luteus* ATCC 10240	3	3	1	3	±	3
5. *Arthrobacter simplex* ATCC 6946	2	3	0	0	0	0
6. *Corynebacterium equi* B-271-1	3	3	0	0	0	0
7. *Mycobacterium phlei* ATCC 6946	2	3	±	±	±	±
D. *Bacillus*:						
1. *B. cereus* IFO 3001	3	3	0	0	0	0
2. *B. cereus* 60-6	3	3	0	0	0	0
3. *B. subtilis* IFO 3007	3	3	0	0	0	0
4. *B. subtilis* AR-30	3	3	0	0	0	0
5. *B. pumilus* TL-47	3	3	0	1	0	1

Table 1. (Continued)

	Tunicamycin					
	0 μg/ml		10 μg/ml		50 μg/ml	
Days	2	6	2	6	2	6
6. *B. licheniformis* ATCC 12199	3	3	0	1	0	0
7. *B. circulans* NRRL 3313	3	3	0	0	0	0
8. *B. laterosporus* 340–19	3	3	0	0	0	0
9. *B. pulvifaciens* CB-57	3	3	0	0	0	0
10. *B. brevis* Ak-4	2	3	0	0	0	0
11. *B. polymyxa* AR-110	2	3	0	0	0	0
12. *B. circulans* Bz-43	2	3	0	0	0	0
13. *B. sphericus* ATCC 7055	3	3	0	2	0	1
E. *Pseudomonas*:						
1. *P. aeruginosa* IFO 3449	3	3	3	3	3	3
2. *P. aeruginosa* IFO 3812	3	3	3	3	3	3
3. *P. chlororaphis* IFO 3506	3	3	3	3	3	3
4. *P. chlororaphis* IFO 3904	3	3	3	3	3	3
5. *P. chlororaphis* ATCC 17810	3	3	3	3	3	3
6. *P. pyrrocinica* ATCC 15958						
7. *P. fluorescens* IFO 3507	3	3	3	3	3	3
8. *P. riboflavinus* IFO 3140	2	3	0	1	0	0
9. *P. syncyanea* IFO 3757	1	3	1	2	1	2
10. *P. fragi* IFO 3458	3	3	2	3	2	3
11. *P. putida* IFO 3738	3	3	3	3	2	3
F. Enterobacteria:						
1. *Bacterium ketoglutaricum* B-4	3	3	3	3	1	2
2. *Escherichia coli* mutaflora	3	3	3	3	2	3
3. *Citrobacter freundii* IRP-S-87	3	3	3	3	1	2
4. *Aerobacter aerogenes* ATCC 8724	3	3	3	3	2	3
5. *Proteus mirabilis* OM-8	2	3	2	2	1	2
6. *Proteus vulgaris* YO-5	3	3	3	3	2	2

a) Assay conditions: As described in "Materials and Methods". 3 signifies good growth, 2 moderate growth, 1 weak growth, ± scanty growth and 0 no growth.

one slow grower, *M. megalomicea* NRRL 3274 which gave good growth only at 10 μg/ml at 10 days (see Table 1). In contrast, all the Gram-negative bacteria tested were insensitive to the tunicamycin at 10 and 50 μg/ml concentrations with one exception, *Ps. riboflavinus* IFO 3140 (see Table 1). The results indicated that tunicamycin is usable for differential isolation of *Micromonospora* from other Gram-positive bacteria.

Optimum Concentration of Tunicamycin

Thirteen strains of well-known micromonosporae were used to find maximal allowable concentrations to tunicamycin, with the results presented in Table 2. Most of the test strains grew at 25 to 50 μg per ml of tunicamycin. *M. megalomicea* NRRL 3718 however showed no growth at 50 μg per ml (but grew at 10 μg per ml). Therefore, the majority of the *Micromonospora* strains will grow and form colonies on the agar medium containing 25 to 50 μg per ml of tunicamycin.

Next, the colony-forming test on the tunicamycin agar was performed. Three *Micromonospora* strains, *M. purpurea* NRRL 2953, *M. echinospora* NRRL 2985 and *Micromonospora* sp. X-365, were enumerated on the agar medium containing 50 and 100 μg per ml of tunicamycin. The conditions and results of these tests are presented in Table 3. *M. echinospora* NRRL 2985 and *M. purpurea* NRRL 2953 formed similar numbers of colonies with both concentrations of tunicamycin-agar and the control (modified BENNET's medium), respectively. *Micromonospora* sp. X-356 recovered 76% and 47% of the colonies against control on the 50 μg and 100 μg per ml plates, respectively (see Table 3). The results suggest that 50 μg or less per ml of tunicamycin is suitable for micromonosporae isolation.

Table 2. Maximal allowable concentrations of tunicamycin to various *Micromonospora* strains[a].

No.	*Micromonospora* tested	MAC of tunicamycin μg per ml
1.	*M. purpurea* NRRL 2953	>50
2.	*M. echinospora* subsp. *inyoensis* NRRL 3292	>50
3.	*M. echinospora* NRRL 2985	25
4.	*M. grisea* NRRL 3800	50
5.	*M. rosaria* NRRL 3718	50
6.	*M. megalomicea* NRRL 3718	10
7.	*M. chalcea* subsp. *flavida* NRRL 3222	>50
8.	*M. carbonacea* NRRL 2972	>50
9.	*M. halophytica* NRRL 3097	>50
10.	*Micromonospora* sp. X-378	>50
11.	*Micromonospora* sp. X-365	>50
12.	*Micromonospora* sp. F-165	>50
13.	*Micromonospora* sp. F-952	>50
14.	*Streptomyces griseus* 5049 B-3	<10
15.	*Streptomyces kasugaensis* ATCC 15714	<10

[a] Assay conditions: As described in "Materials and Methods". Modified BENNET's agar medium was used in this assay.

Attempted Isolation of *Micromonospora* from 400 Soil Samples

In order to test whether the tunicamycin medium is practically usable for routine isolation of *Micromonospora,* an attempted isolation was performed with 400 soil samples from various regions around the world. Modified BENNET's medium containing 25 μg/ml of tunicamycin and 30 μg/ml of cycloheximide was used in this test. The results are presented in Table 4.

Many *Micromonospora* colonies were detected from most plates. All samples gave *Micromonospora* colonies on this agar medium. The colonies, which differed in appearance, were isolated from each soil sample (Initial Isolates). The contaminated strains other than *Micromonospora* in the Initial Isolates were eliminated by microscopic checking, and appearance of the remaining Initial Isolates from a soil sample were compared on agar slants, and the strains having identical appearance were

Table 3. Enumerations of three *Micromonospora* strains on tunicamycin agar[a].

	Tunicamycin μg/ml	Number of colonies		Tunicamycin μg/ml	Number of colonies	
		Exp. 1	Exp. 2		Exp. 1	Exp. 2
1. *M. purpurea* NRRL 2953	0	366	372	0	83	86
	50	348	359	100	85	76
2. *M. echinospora* NRRL 2985	0	195	225	0	79	72
	50	198	186	100	70	56
3. *Micromonospora* sp. X-365[b]	0	513	542	0	906	903
	50	401	396	100	433	414

[a] The enumerations were performed in doublet by modified BENNET's agar medium with or without tunicamycin. Incubated at 28°C for two weeks.

[b] Incubated at 28°C for four weeks.

Table 4. Attempted isolation of *Micromonospora* from 400 soil samples[a].

No.	Soil number	From	Initial Isolates	Selected Isolates[b]	Selected/ soil
1.	95	Wakayama district, Japan	300	202	2.13
2.	68	Okinawa district, Japan	585	287	4.22
3.	81	Australia (Queensland, Newsouthwales, Victoria & Tasmania)	726	381	4.70
4.	26	New Zealand	402	135	5.19
5.	49	Malaysia	435	236	4.82
6.	41	Burma	242	154	3.76
7.	40	Neigeria, Cameroun & Gabon	307[c]	190	4.75

[a] Conditions for isolation: Modified BENNET's agar medium containing 25 μg/ml of tunicamycin and 30 μg/ml of cycloheximide. Incubated at 28°C for 2 to 4 weeks.

[b] The similar appearing strains from same the soil sample were handled as one strain.

[c] Contained 22 strains of contaminants (bacteria and *Streptomyces*) (see Results).

grouped as one strain (Selected Isolates). For example, 307 strains of Initial Isolates from *Neiseria* Cameroun and Gabon Soils (see Table 4) contained 18 strains of *Streptomyces* (no aerial mycelium type), one of *Nocardia* strain, three strains of rod-shaped bacteria, and 95 strains of repeated isolates of *Micromonospora*. A total 1585 strain of Selected Isolates of *Micromonospora* were obtained from 2997 strains of Initial Isolates from the 400 soil samples. On the assumption that all the Selected Isolates differented in each other (see next section), about 4 different *Micromonospora* strains could be isolated from a soil sample by this procedure. The results indicate that the tunicamycin method is practical and usable for antibiotic screening of *Micromonospora*.

Diversity of the "Selected Isolates"

To inspect the diversity of Selected Isolates, 98 strain of Selected Isolates from Neiseria, Cameroun and Gabon soils were analyzed for their taxonomic characters and antibiotic productivities. The 27 differential keys including 22 taxonomic characters and six antibiotic productivities were used in this test (see Table 5). All 98 strains differed from each other in their taxonomic character(s) and/or antibiotic productivities (see Table 5). The results indicate that most, if not all, the Selected Isolates of *Micromonospora* are different in each other.

Elimination of Gram-negative Contaminants in the Sample

Tunicamycin inhibits the growth of Gram-positive bacteria, except *Micromonospora*, but does not affect on Gram-negative bacteria. In some cases, colonies of Gram-negative contaminants from a soil sample interfered with the isolation of *Micromonospora*. To reduce the Gram-negative contaminants, the samples were subjected to alkaline treatment (see Materials and Methods).

Survival tests of 98 strains of various Gram-negative and -positive bacteria in 0.01 N NaOH were done at about 15°C for 5 to 180 minutes, with the results shown in Table 6. Most tested strains of Gram-negative bacteria were dead with 5 minutes treatment in 0.01 N NaOH solution, while most Gram-positive bacteria including *Micromonospora* survived for the 5 to 30 minutes treatment. The results clearly show that alkaline treatment of the sample with 0.01 N NaOH for 5 to 10 minutes at about 15°C is useful for eliminating the Gram-negative contaminants.

Table 5. Diversity of a group of the Selected Isolates on their taxonomic characters and/or antibiotic-productivities.

Color of cell paste	Strain No.	Spore surface	Soluble pigment	Melanoid	Tyrosinase	Acid from glucose	NO_2 from NO_3	Milk-peptonization	Milk-coagulation	Gelatin-liquefaction	Starch utilization	Arabinose utilization	Xylose utilization	Glucose utilization	Fructose utilization	Sucrose tuilization	*m*-Inositol utilization	Rhamnose utilization	Raffinose utilization	Mannitol utilization	Melibiose utilization	Gp-active on MG_4[a]	Gn-active on MG_4	Y-active on MG_4	Gp-active on BENNET'S[a]	Gn-active on BENNET'S	Y-active on BENNET'S
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. Pale yellowish brown	1	Smooth	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	+	−	+	+	−	−	−	−	−
2. Pale yellowish brown	5	Smooth	−	−	−	−	±	+	−	+	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−
3. Pale yellowish brown	16	Smooth	−	−	−	−	±	+	−	−	+	±	+	+	±	+	−	−	+	−	+	−	−	−	−	−	−
4. Pale yellowish brown	19	Smooth	−	−	−	−	+	+	−	−	+	−	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
5. Pale yellowish brown	39	Smooth	−	−	−	−	+	+	−	−	+	−	+	+	−	+	−	+	+	−	−	−	−	−	−	−	−
6. Pale yellowish brown	8	Smooth	−	−	−	−	−	−	−	−	+	−	+	+	±	+	−	+	+	−	+	−	−	−	−	−	−
7. Pale yellowish brown	20	~[b]	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−
8. Pale yellowish brown	76	Smooth	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	+	−	+	−	+	+	+	+	−	−
9. Pale yellowish brown	71	Smooth	−	~	−	−	−	−	−	−	−	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
10. Pale yellowish brown	27	Smooth	−	−	−	−	±	−	−	−	+	±	+	+	±	+	−	±	+	−	+	+	−	−	−	−	−
11. Pale yellowish brown	54	Smooth	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
12. Pale yellowish brown	81	Smooth	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−
13. Yellowish brown	9	Smooth	+	−	−	−	+	+	−	−	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
14. Yellowish brown	84	Smooth	+	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	+	−	+	−	+	−	−	−
15. Yellowish brown	100	Smooth	+	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	+	+	+	−	−	−	−	−	−
16. Yellowish brown	50	Smooth	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+	+	±	−	−	−	−	−
17. Yellowish brown	26	Smooth	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−	−	−
18. Yellowish brown	68	Smooth	−	−	−	−	−	+	−	−	−	+	−	+	+	+	−	+	+	+	+	+	−	−	−	−	−
19. Yellowish brown	22	Warty	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	±	+	−	−	−	−	−	−
20. Yellowish brown	29	Warty	−	−	−	−	−	−	±	+	+	+	+	+	+	+	−	+	+	−	+	−	−	−	+	−	−
21. Yellowish brown	48	Warty	−	−	−	−	−	+	+	+	+	−	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−
22. Yellowish brown	77	Warty	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	−	−	+	−	−
23. Yellowish brown	34	Warty	−	−	−	−	±	−	−	−	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	+

24.	Yellowish brown	7	~	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−
25.	Light brown	25	Smooth	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26.	Light brown	36	Smooth	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−
27.	Light brown	70	Smooth	+	−	−	−	−	+	−	+	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
28.	Light brown	89	Warty	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
29.	Light brown	60	~	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
30.	Brown	31	Smooth	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
31.	Brown	98	Smooth	+	−	−	−	−	+	−	−	−	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−
32.	Dark brown	33	Smooth	+	−	−	−	−	+	−	−	−	+	+	+	±	+	−	−	−	+	−	+	−	−	−	−	+
33.	Dark brown	47	Smooth	+	−	−	−	−	+	−	+	~	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−
34.	Dark brown	52	Smooth	+	−	−	−	−	+	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	−	−	−	−
35.	Dark brown	66	Smooth	+	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−
36.	Dark brown	69	Smooth	−	−	−	−	−	+	−	−	−	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	−
37.	Dark brown	55	Warty	+	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	−	−	−	−	−
38.	Dark brown	64	Warty	+	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−	−
39.	Dark brown	41	Blunt spines	+	~	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+
40.	Olive gray	4	Smooth	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
41.	Olive gray	38	Smooth	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−
42.	Dark olive gray	78	Smooth	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
43.	Dark brown gray	14	Smooth	+	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−
44.	Dark purplish gray	24	Smooth	+	−	−	−	−	−	−	−	+	+	−	±	±	±	−	−	±	−	−	+	−	−	+	−	−
45.	Dark purplish gray	15	Blunt spines	+	~	−	−	−	+	−	−	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
46.	Purplish gray	83	Blunt spines	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
47.	Light organge	3	Smooth	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
48.	Yellowish orange	28	Smooth	−	−	−	+	−	−	−	−	+	−	±	±	±	±	±	±	±	−	+	−	−	−	−	−	−
49.	Yellowish orange	40	Smooth	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	±	+	−	−	−	−	−	−	−
50.	Yellowish orange	43	Warty	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
51.	Orange	32	Smooth	−	−	−	−	±	−	−	−	−	±	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−
52.	Orange	37	Smooth	−	−	−	+	+	−	−	+	+	−	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−
53.	Orange	58	Smooth	−	−	−	−	−	−	−	−	−	+	+	+	−	±	−	+	+	−	+	−	−	−	−	−	−
54.	Orange	80	Smooth	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	−

Table 5. (Continued).

Color of cell paste	Strain No.	Spore surface	Soluble pigment	Melanoid	Tyrosinase	Acid from glucose	NO_2 from NO_3	Milk-peptonization	Milk-coagulation	Gelatin-liquefaction	Starch utilization	Arabinose utilization	Xylose utilization	Glucose utilization	Fructose utilization	Sucrose utilization	*m*-Inositol utilization	Rhamnose utilization	Raffinose utilization	Mannitol utilization	Melibiose utilization	Gp-active on MG_4[a)]	Gn-active on MG_4	Y-active on MG_4	Gp-active on BENNET's[a)]	Gn-active on BENNET's	Y-active on BENNET's
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
55. Orange	85	Smooth	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−
56. Orange	72	~	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	+	+	+	−	−	−	−	−	−
57. Dark orange	45	Smooth	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−
58. Purplish orange	93	Warty	−	−	−	−	−	+	−	−	−	+	+	+	+	+	−	−	+	−	+	−	−	−	+	−	−
59. Olive black	86	Blunt spines	+	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−
60. Olive black	90	Warty	−	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
61. Brown black	2	Warty	+	−	−	−	−	+	−	−	+	−	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
62. Brown black	30	Warty	+	−	−	−	+	+	−	+	+	+	+	+	+	+	−	−	+	±	+	+	−	−	−	−	−
63. Brown black	46	Warty	+	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−
64. Brown black	79	Warty	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	+	−	+	−	+	−	−	−	−	−
65. Brown black	90	Warty	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
66. Brown black	53	Warty	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
67. Brown black	59	Warty	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	+	−	−
68. Brown black	62	Warty	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−
69. Brown black	35	Smooth	+	~	−	−	±	−	−	−	+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	−	−
70. Brown black	44	Smooth	+	−	−	−	−	+	−	+	−	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−
71. Brown black	63	Smooth	+	−	−	−	−	−	−	+	−	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
72. Brown black	65	Smooth	+	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	−	±	−	−
73. Brown black	67	Smooth	+	~	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
74. Brown black	94	Smooth	+	−	−	−	−	+	−	−	−	+	+	+	+	+	−	−	−	−	+	−	−	−	+	+	+
75. Brown black	87	Smooth	−	−	−	−	−	+	−	−	+	+	+	+	−	−	−	−	−	+	−	+	−	−	−	−	−
76. Brown black	97	Smooth	−	−	−	−	−	+	−	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−

77.	Purplish black	12	Warty	+	−	−	−	−	+	−	−	+	−	+	+	−	+	−	−	+	−	+	+	−	−	−	−	−
78.	Purplish black	75	Warty	+	−	−	−	+	+	−	+	+	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
79.	Purplish black	17	Smooth	+	−	−	−	±	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
80.	Purplish black	18	Smooth	+	−	−	−	±	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
81.	Purplish black	57	Smooth	+	−	−	−	−	+	−	+	+	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
82.	Blueish black	61	Warty	+	−	−	−	−	−	−	−	+	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
83.	Blueish black	82	Smooth	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
84.	Greenish black	13	Smooth	+	−	−	+	±	−	−	−	~	−	−	−	−	±	−	−	±	−	−	+	−	−	−	−	−
85.	Greenish black	56	Smooth	+	−	−	−	−	+	−	+	+	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−
86.	Greenish black	51	Smooth	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
87.	Greenish black	42	Blunt spines	+	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−	−	+	+	+	−	−	−
88.	Black	10	Warty	+	−	−	−	−	+	−	+	+	−	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
89.	Black	11	Warty	+	−	−	−	−	+	−	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−
90.	Black	95	Warty	+	−	−	+	+	+	−	−	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
91.	Black	99	Warty	+	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−
92.	Black	49	Warty	−	+	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−
93.	Black	74	Smooth	+	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+	−	+	+	−	+	−	−	−
94.	Black	73	Smooth	−	−	−	−	−	+	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−
95.	Black	91	Smooth	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	+	+	−	−	−
96.	Black	88	Blunt spines	+	−	−	−	−	+	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
97.	Purple	92	Blunt spines	+	−	−	−	−	−	−	−	+	−	−	±	−	−	−	−	−	−	−	+	+	+	−	−	−
98.	Dark purple	6	Smooth	+	−	−	−	+	+	−	−	+	+	+	+	+	+	−	−	+	−	+	−	−	−	+	−	−

a) Antibiotic productivities of the Selected Isolates were determined by agar-disc method. The agar discs of MG_4 and modified BENNET's media containing the growth of *Micromonospora* (2~3 days and 7 days stages at 28°C) were placed on agar plates seeded with the test microoganisms, and the antibiotic activities were measured after 16 to 20 hours incubation at 37°C. *Staphylococcus aureus* 209p JC-1 (Gp), *Escherichia coli* 209p JC-2 (Gn) and *Candida albicans* M-9 (Y) were used as the test organisms. The MG_4 medium has following composition: 2.0% glucose, 0.5% glycerol, 1.5% Soytone (Difco), 0.5% corn steep liquor, 0.3% $CaCO_3$, 0.3% NaCl and 1.5% agar.

b) not measured.

Table 6. Alkaline treatment of various Gram-positive and negative bacteria[a].

	Survival ratio %			
	0 minute	5 minutes	10 minutes	30 minutes
A. *Pseudomonas*:				
1. *P. aeruginosa* IFO 3455	100	0	0	0
2. *P. aeruginosa* IFO 3812	100	0	0	0
3. *P. aeruginosa* Denken	100	0	0	0
4. *P. chlororaphis* IFO 3506	100	0	0	0
5. *P. chlororaphis* IFO 3904	100	0	0	0
6. *P. syncyanea* IFO 3757	100	0	0	0
7. *P. fluorescens* ATCC 17810	100	0	0	0
8. *P. fluorescens* IFO 3507	100	0	0	0
9. *P. fragi* IFO 3458	100	0	0	0
B. *Acetobacter*:				
1. *A. albidus* IFO 3250	100	0	0	0
2. *A. fragum* BK-P-N863	100	0	0	0
3. *A. gluconicus* IFO 3171	100	0	0	0
4. *A. industrium* IFO 3260	100	24.8	0	0
5. *A. melanogenes* IFO 3293	100	0	0	0
6. *A. suboxydans* ATCC 612	100	0	0	0
7. *A. xylinum* IFO 3288	100	0	0	0
C. *Vibrio*:				
1. *V. percolans* ATCC 8461	100	0	0	0
D. Escherichieae:				
1. *E. coli* 209P JC-2	100	0	0	0
2. *C. freundii* IRP-S87	100	0	0	0
E. Aerobacter:				
1. *A. aerogenes* ATCC 8724	100	0	0	0
2. *A. hibernicus* S-5	100	88.0	83.0	0
F. *Proteus*:				
1. *P. vulgaris* YO-5	100	0	0	0
2. *P. mirabilis* IRP-OM-8	100	9.9	0	0
3. *P. rettgerii* IRP-OR-6	100	0	0	0
G. *Salmonella*:				
1. *S. typhimurium* TA-1535	100	0	0	0
2. *S. typhimurium* TA-98	100	0	0	0
H. *Alkaligenes*:				
1. *A. metalkaligenes* AN-23	100	0	0	0
I. *Staphylococcus*:				
1. *S. aureus* 209P JC-1	100	81.5	45.3	59.0
J. *Micrococcus*:				
1. *M. candidus* E-28-1031	100	98.5	97.5	61.2
2. *M. citreus* IFO 3332	100	74.7	16.8	2.3
3. *M. luteus* ATCC 10240	100	78.7	71.1	59.0

Table 6. (Continued).

	Survival ratio %			
	0 minute	5 minutes	10 minutes	30 minutes
4. *M. freudenreichii* A-57	100	0	0	0
5. *M. lysodeikticus* IFO 3333	100	71.1	51.3	39.5
6. *M. roseus* IFO 3764	100	30.1	18.8	12.4
K. *Corynebacterium*:				
1. *C. carnae* NRRL B2233	100	99.4	85.8	83.3
2. *C. equi* B-271-1	100	28.4	28.2	1.0
3. *C. simplex* IFO 3530	100	0	0	0
L. *Brevibacterium*:				
1. *B. ammoniagenes* ATCC 6871	100	98.8	97.7	75.2
2. *B. imperiale* ATCC 8363	100	92.5	88.4	98.8
3. *B. linens* ATCC 9172	100	91.3	79.1	66.2
M. *Arthrobacter*:				
1. *A. citreus* ATCC 11624	100	25.9	16.0	0
2. *A. globiformis* ATCC 8010	100	1.3	1.0	0
3. *A. simplex* ATCC 6946	100	3.0	0.5	0
4. *A. tumescens* ATCC 6947	100	70.8	68.8	24.6
N. *Microbacterium*:				
1. *M. flavum* OJ-9	100	62.2	47.7	25.7
2. *M. lacticum* S-2 Maryland	100	88.9	74.1	78.4[a)]
O. *Bacillus*:				
1. *B. brevis* IFO 3331	100			95.4
2. *B. cereus* IFO 3001	100			41.3
3. *B. circulans* NRRL B3313	100	99.3	98.8	
4. *B. licheniformis* IFO 12199	100			88.7
5. *B. megatherium* IFO 1035	100			96.6
6. *B. polymyxa* IFO 3020	100			57.2
7. *B. sphericus* ATCC 7055	100			48.8
8. *B. pumilus* IFO 3813	100			92.0
9. *B. subtilis* IFO 3007	100			75.3
P. *Mycobacterium*:				
1. *M. avium* IFO 3082	100			34.2
2. *M. avium* 607	100			54.9
3. *M. phlei* ATCC 19249	100			33.7
4. *M. phlei* IFO 3158	100			36.1
5. *M. smegmatis* IFO 3085	100			31.8
Q. *Nocardia*:				
1. *N. asteroides* E, H. Drake	100			36.0
2. *N. asteroides* NIH 9935	100			38.7
3. *N. asteroides* ITK 205	100			18.9
4. *N. blackwallii* ATCC 8646	100			40.0
5. *N. blasiliensis* Momillan O-416	100			58.4
6. *N. blasiliensis* ITK 195	100			26.8

Table 6. (Continued).

	Survival ratio %			
	0 minute	5 minutes	10 minutes	30 minutes
7. *N. coeliaca* ITK 122	100			77.1
8. *N. eppingeri* Pasteur Inst. No. 508	100			8.9
9. *N. erythropolis* Waksman 3407	100			47.1
10. *N. leichimanii* ATCC 6855	100			21.9[a]
11. *N. lutea* ITK 192	100			68.7
12. *N. madura* No. 333	100			31.8
13. *N. mexicana* SRL 5219	100			41.4
14. *N. polychromogenes* Jensen CBS	100			39.6
15. *N. transvalensis* ITK 193	100	91.5	90.5	67.6
16. *N. uniformis* var. *tsuyamanensis* ATCC 21806	100	37.7	18.6	0.3
R. *Streptomyces*:				
1. *S. cattleya* NRRL 8057	100			79.6
2. *S. erythraeus* F-188	100	22.7	21.5	14.1
3. *S. fradiae* Waksman 3535	100	40.7	38.0	29.0
4. *S. griseus* NRRL 3851	100			76.4
5. *S. humidus* ATCC 12760	100			86.3
6. *S. kanamyceticus* ATCC 12853	100	75.5	73.4	54.1
7. *S. kasugaensis* ATCC 12853	100	58.0	53.8	24.2
8. *S. lactamdurans* NRRL 3802	100	56.9	42.5	1.7
9. *S. levoris* ATCC 5876	100			70.5
10. *S. lincolnensis* NRRL 2936	100	72.7	50.0	31.2
11. *S. ribosidificus* ATCC 21294	100	98.0	85.7	83.7
12. *S. vinaceus* NRRL B2285	100			64.1
13. *S. lavendulae* K433	100	68.9	55.9	
14. *S. antibioticus* E622	100			97.4
15. *S. hygroscopicus* K775	100	27.9	17.1	1.8
S. *Micromonospora*:				
1. *M. carbonacea* NRRL 2972	100	61.7	56.8	55.7
2. *M. chalcea* subsp. *flavida* NRRL 3222	100	87.1	82.2	71.8
3. *M. echinospora* NRRL 2985	100	95.2	79.4	79.4
4. *M. echinospora* subsp. *inyoensis* NRRL 3292	100	92.1	85.1	79.9
5. *M. halophytica* subsp. *niger* NRRL 3092	100	87.2	65.4	31.7
6. *M. purpurea* NRRL 2953	100	100	94.1	68.2
7. *M. rosaria* NRRL 3718	100	72.7	25.1	3.3

[a] Incubated at 37°C for CFU counting.

Discussion

Tunicamycin[20] is a nucleoside antibiotic from *Streptomyces lysosuperificus* and is active against RNA viruses, Gram-positive bacteria and some fungi and yeast. TAKATSUKI, *et al.*[21] and TKACZ and LAMPEN[22] found separately that tunicamycin selectively inhibits the formation of polyisoprenyl-*N*-acetylglucosamine phosphate in the cell-free microsomal system of chick embryos and calf liver, respectively. BETTINGER and YOUNG[23] demonstrated that the antibiotic blocked transfer of *N*-acetyl-

glucosaminylphosphate from UMP directly onto undecaprenylphosphate in *Bacillus* peptidoglycan synthesis. SASAKI, *et al.*[24] reported that this antibiotic inhibited the formation of UMP-*N*-acetylmuramylpentapeptide and lipid. KUO and LAMPEN[25] also found it inhibited the incorporation of glucosamine into yeast glycoprotein. These results support the hypothesis that tunicamycin inhibits a step(s) of peptidoglycan synthesis which operates in Gram-positive bacteria, yeast and animals but not in Gram-negative bacteria and *Micromonospora*. *Micromonospora* are known to be Gram-positive bacteria[26] but form many phenazine antibiotics resembling to these from *Pseudomonas*[11].

The tunicamycin enriched the isolation of *Micromonospora* by inhibiting growth of various Gram-positive bacteria other than *Micromonospora*. A majority of *Micromonospora* strains, if not all, could be isolated vary easily and efficiently from soil samples with an isolation medium containing 25 to 50μg per ml of tunicamycin. Alkaline treatment (0.01 N NaOH, 5~10 minutes at 15°C) was useful for the soil samples containing much Gram-negative bacteria.

Using the tunicamycin method, 1,585 strains of Selected Isolates of *Micromonospora* were obtained from 400 soil samples collected from various regions around the world. They would be different from each other since the sampling inspection of 98 Selected Isolates gave different results on their taxonomic character(s) and/or antibiotic productivities. Therefore, about four different strains of *Micromonospora* could be isolated, in average, from each soil sample. Our results suggests that tunicamycin method makes possible a simple, concentrated screening method for new antibiotic from *Micromonospora*.

Most of the 400 soil samples contained *Micromonospora*. As SOLOVIEVA and SINGAL have stated[15], *Micromonospora* strains were distributed widely in soils of various geographical regions. They were, however, not predominant in silts in our tests (data not shown).

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References

1) ROSSELET, J. P.; J. A. MARQUEZ, E. MESECK, A. MURAWSKI, A. HAMDAN, C. JOYNER, R. SCHMIDT, D. MIGLIORE & H. L. HERZOG: Isolation, purification and characterization of gentamicins. Antimicrob. Agents Chemother. 1963: 14~16, 1964
2) WEINSTEIN, M. J.; J. A. MARQUEZ, R. T. TESTA, G. H. WAGMAN, E. M. ODEN & J. A. WAITZ: Antibiotic 6640, a new *Micromonospora*-produced aminoglycoside antibiotic. I. J. Antibiotics 23: 551~554, 1970
WAGMAN, G. H.; R. T. TESTA & J. A. MARQUEZ: Antibiotic 6640. II. Fermentation, isolation and properties. J. Antibiotics 23: 555~558, 1970
3) WAGMAN, G. H.; R. T. TESTA, J. A. MARQUEZ & M. J. WEINSTEIN: Antibiotic G-418, a new *Micromonospora*-produced aminoglycoside with activity against *Protozoa* and *Hilminths*: Fermentation, isolation and preliminary characterization. Antimicrob. Agents Chemother. 6: 144~149, 1974
4) WEINSTEIN, M. J.; G. H. WAGMAN, J. A. MARQUEZ, R. T. TESTA & J. A. WAITZ: Verdamicin, a new broad spectrum aminoglycoside antibiotic. Antimicrob. Agents Chemother. 7: 246~249, 1975
5) OKACHI, R.; I. KAWAMOTO, S. TAKASAWA, M. YAMAMOTO, S. SATO, T. SATO & T. NARA: A new antibiotic XK-62-2 (Sagamicin). I. Isolation, physicochemical and antibacterial properties. J. Antibiotics 27: 793~800, 1974
6) MARQUEZ, J. A.; G. H. WAGMAN, R. T. TESTA, J. A. WAITZ & M. J. WEINSTEIN: A new broad spectrum aminoglycoside antibiotic, G-52, produced by *Micromonospora zionensis*. J. Antibiotics 29: 483~487, 1976
7) WEINSTEIN, M. J.; G. H. WAGMAN, J. A. MARQUEZ, R. T. TESTA, E. ODEN & J. A. WAITZ: Megalomicin, a new macrolide antibiotic complex produced by *Micromonospora*. J. Antibiotics 22: 253~258, 1969
8) WAGMAN, G. H.; J. A. WAITZ, J. A. MARQUEZ, A. MURAWSKI, E. M. ODEN, R. T. TESTA & M. J. WEINSTEIN: A new *Micromonospora*-produced macrolide antibiotic, rosamicin. J. Antibiotics 25: 641~646, 1972

9) KAWAMOTO, I.; R. OKACHI, H. KATO, S. YAMAMOTO, I. TAKAHASHI, S. TAKASAWA & T. NARA: The antibiotic XK-41 complex. I. Production, isolation and characterization. J. Antibiotics 27: 493~501, 1974
10) FURUMAI, T.; I. MAEZAWA, N. MATSUZAWA, S. YANO, T. YAMAGUCHI, K. TAKEDA & T. OKUDA: Macrolide antibiotics M-4365 produced by *Micromonospora*. I. Taxonomy, production, isolation, characterization and properties. J. Antibiotics 30: 443~449, 1977
11) GERBER, N. N. & M. P. LECHEVALIER: Phenazines and phenoxazinones from *Micromonospora aerata* sp. nov. and *Pseudomonas iodina*. Biochemistry 3: 598~602, 1964
12) PAGANI, H.; G. LANCINI, G. TAMONI & C. CORONELLI: Tetrenolin and SS/1018 A, antimicrobial agents isolated from a strain of Actinomycetales. J. Antibiotics 26: 1~6, 1973
13) HATANO, K.; E. HIGASHIDE & M. SHIBATA: Studies of juvenimicin, a new antibiotic. I. Taxonomy, fermentation and antimicrobial properties. J. Antibiotics 29: 1163~1170, 1976
KISHI, T.; S. HARADA, H. YAMANA & A. MIYAKE: Studies of juvenimicin, a new antibiotics. II. Isolation, chemical characterization and structures. J. Antibiotics 29: 1171~1181, 1976
14) NAKAMURA, S.; T. CHIKAIKE, K. KARASAWA, N. TANAKA, H. YONEHARA & H. UMEZAWA: Isolation and characterization of bottromycins A and B. J. Antibiotics, Ser. A 18: 47~52, 1965
15) SOLOVIEVA, N. K. & E. M. SINGAL: Some data on ecology of *Micromonospora*. Antibiotiki 17: 778~781, 1972
16) SINGAL, E. M.; L. P. IVANITSUKAIA, M. P. SMIRNOVA & S. M. NAVASHIN: Direct isolation of *Micromonospora* cultures from moist soils and silts. Antibiotiki 23: 693~696, 1978
17) NONOMURA, H. & Y. OHARA: Distribution of Actinomycetes in soil. IV. Isolation and taxonomy of *Microbispora*. J. Ferment. Technol. 38: 401~405, 1960
18) NONOMURA, H. & Y. OHARA: Distribution of Actinomycetes in soil. VI. A culture method effective for both preferential isolation and enumeration of *Microbispora* and *Streptosporangium* strains in soil. I. J. Ferment. Technol. 47: 463~469, 1969
19) IVANITSKAYA, L. P.; S. M. SINGAL, M. V. BIBIKOVA & S. N. VOSTROV: Direct isolation of *Micromonospora* on selective media with gentamicins. Antibiotiki 23: 690~692, 1978
20) TAKATSUKI, A.; K. ARIMA & G. TAMURA: Tunicamycin, a new antibiotic. I. Isolation, and characterization of tunicamycin. J. Antibiotics 24: 215~223, 1971
21) TAKATSUKI, A.; K. KOHNO & G. TAMURA: Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agric. Biol. Chem. 39: 2089~2091, 1975
22) TKACZ, J. S. & J. O. LAMPEN: Tunicamycin inhibition of polyisoprenyl-*N*-actylglucosaminyl pyrrophosphate formation in calf-liver microsomes. Biochem. Biophys. Res. Commun. 65: 248~257, 1975
23) BETTINGER, G. E. & F. E. YOUNG: Tunicamycin, a inhibitor of *Bacillus* peptidoglycan synthesis, new site of inhibition. Biochem. Biophys. Res. Commun. 67: 16~21, 1975
24) TAMURA, G.; T. SASAKI, M. MATSUHASHI, A. TAKATSUKI & M. YAMASAKI: Tunicamycin inhibits the formation of lipid intermediates in cell-free peptidoglycan synthesis of bacteria. Agric. Biol. Chem. 40: 447~449, 1976
25) KUO, S. O. & J. O. LAMPEN: Tunicamycin inhibition of tritium-laveled glucosamine incorporation into yeast glycoproteins: Binding of tunicamycin and interaction with phospholipids. Arch. Biochem. Biophys. 172: 574~581, 1976
26) BUCHANAN, R. E. & N. E. GIBBONS (*ed*): BERGEY's Manual of Determinative Bacteriology. 8th ed., The Williams and Wilkins Company, Baltimore, 1974