A PREFERENTIAL ISOLATION PROCEDURE FOR ASPOROGENOUS GRAM-POSITIVE BACTERIA

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(Received for publication October 26, 1981)

A preferential isolation procedure was devised for asporogenous (Asp), Gram-positive (Gp), aerobic or facultative anaerobic bacteria which included the genera *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Microbacterium*, *Mycobacterium*, and *Micrococcus* (Asp-Gp bacteria). An antibiotics-mixture agar which contained 5 to 10 μg per ml of colistin, 10 to 20 μg per ml of nalidixic acid and 30 μg per ml of cycloheximide was used in the isolation. Using this technique 47 Asp-Gp bacteria representing 26 subgroups of coryneform bacteria and *Micrococcus* were isolated from 3 soil samples. The method was far more efficient than the standard dilution-plate technique. This preferential method is available to isolate Asp-Gp bacteria from a sample containing about 500-fold more of other Gram-positive and negative bacteria.

The asporogenous Gram-positive group of aerobic or facultative anaerobic bacteria (Asp-Gp bacteria) includes the genera *Arthrobacter, Corynebacterium, Brevibacterium, Microbacterium, Cellulomonas, Kurthia, Mycobacterium,* and *Micrococcus* and is taxonomically close to *Streptomycetaceae* without *Micrococcus*.[1] The Asp-Gp bacteria also resemble *Streptomycetaceae* in antibiotic productivity. For example, oxamicetin (resembling amicetin of *Streptomyces*[2]) is produced by *Arthrobacter oxamicetes*,[3~5] corynecins (chloramphenicol analogues) by a *Corynebacterium* strain,[6] erythromycin by *Arthrobacter* spp.[7] and hygromycin and epihygromycin by *Corynebacterium equi*.[8] The Asp-Gp bacteria are therefore an important source for new antibiotics as well as *Streptomycetaceae, Bacillaceae* and *Pseudomonadaceae*.

Various kinds of selective or enrichment isolation procedures have been reported for the isolation of Gram-negative bacteria[9~15] and *Streptomycetaceae*[16~24] but no suitable method is available for the isolation of Asp-Gp-bacteria. MILLER and NEVILLE[25] recently devised a medium which was satisfactory for selectively isolating *Arthrobacter* spp. from a variety of soils. They recommended modified Hagedorn and Holt medium (SMHH) with 25 μg/ml of methyl red at neutral pH for the purpose.

This study was initiated to establish a preferential isolation procedure for Asp-Gp bacteria, some of which might be good antibiotic producers. We examined preliminary about 35 kinds of antibiotics for their activity against various Gram-positive and negative bacteria. The results indicated that the mixture of nalidixic acid and colistin at appropriate concentrations might be useful for enrichment isolation of the Asp-Gp bacteria from soil samples.

Materials and Methods

Bacteria Used

Sixty-three strains of Gram-positive and negative bacteria, including *Streptomyces* and *Micromonospora* (see Table 1), were used in sensitivity tests against nalidixic acid and colistin. Most of the strains were obtained from the Institute of Fermentation, Osaka (IFO), the Northern Regional Research

Laboratories, U.S.A. (NRRL) and American Type Culture Collection (ATCC). The others were isolated from soil samples from various districts around the world.

"Asp-Gp-shifted" Isolation Procedure

The enrichment isolation of Asp-Gp bacteria was performed by the following process. About 0.5 g of fresh soil sample was suspended in 5 ml of sterile saline containing 0.01% w/v $MgSO_4 \cdot 7H_2O$ and stirred vigorously with 2~3 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. Next, the suspension was placed in a vacuum desiccator for about 30 minutes in order to eliminate air from the mixture. The vacuum-treated sample was then diluted serially to $10^{-2} \sim 10^{-5}$ and 0.5 ml of each dilution was plated into 15 ml of Gly-IM agar medium which contained 5 μg/ml of colistin, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide. The inoculated agar plates were incubated at 28°C for 1 to 30 days in a moist chamber, and the resulting colonies were isolated. Most Asp-Gp bacteria formed detectable colonies during one week incubation but some strains, including saprophytic strains of *Mycobacterium*, required prolonged incubation. All antibiotics used were sterilized by filtration. Gly-IM agar medium has the following composition: 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% Bactosoytone (Difco), 0.3% NaCl and 1.25% Bacto-agar (Difco), pH 6.8.

Results

Sensitivity Tests of Various Gram-positive and Negative Bacteria to Nalidixic Acid and Colistin

As shown in Table 1, sixty-three strains of Gram-positive and negative bacteria, which included the genera *Pseudomonas, Bacillus, Streptomyces* and *Micromonospora* as well as enterobacteria and Asp-Gp bacteria, were tested for their sensitivities to nalidixic acid and colistin by agar dilution method.

In general, Asp-Gp bacteria, *Pseudomonas*, *Micromonospora* and *Streptomyces* strains were insensitive to 10 and 50 μg/ml of nalidixic acid. In contrast, enterobacteria and *Bacillus* strains tested were sensitive to 50 μg/ml of nalidixic acid and, with the exception of *B. sphaericus* ATCC 7055 and *Proteus mirabilis* OM-8, to 10 μg/ml of the antibiotic. These results indicate that the addition of 10 μg/ml of nalidixic acid to the isolation medium will reduce colonization by both *Bacillus* and enterobacteria to acceptable levels.

Most of *Pseudomonas* strains tested were susceptible to 5 and 25 μg/ml of colistin except for *Pseudomonas riboflavinus* IFO 3140, *Pseudomonas fluorescens* IFO 3507, and *Pseudomonas pyrrocinica* ATCC 15958.[26] All enterobacteria tested were also sensitive to both 5 and 25 μg/ml of colistin with one exception, *Proteus vulgaris* YO-5 which is resistant to both concentrations of the antibiotic. On the other hand, Asp-Gp bacteria were sensitive in general to 25 μg/ml of colistin but all were insensitive to 5 μg/ml. This sensitivity-difference was used to obtain Asp-Gp bacteria.

In general, *Bacillus* and *Streptomyces* were insensitive to 5 μg/ml of colistin but some of them were sensitive to 25 μg/ml of the antibiotic. All *Micromonospora* strains were sensitive against 25 μg/ml of colistin but not all to 5 μg/ml.

From the above results, it appeared that Asp-Gp bacteria could be preferentially isolated by adding 5 μg/ml of colistin in combination with more than 10 μg/ml of nalidixic acid to the isolation medium.

Optimum Concentrations of Nalidixic Acid and Colistin

Using 18 strains of typical Gram-positive and negative bacteria (see Table 2), the optimum concentrations of nalidixic acid and colistin needed to isolate Asp-Gp bacteria and minimize the number

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

		Control		Nalidixic acid				Colistin			
				10 μg/ml		50 μg/ml		5 μg/ml		25 μg/ml	
		2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days
A *Pseudomonas*	1 *P. aeruginosa* IFO 3449	3	3	2	3	0	3	0	0	0	0
	2 *P. aeruginosa* IFO 3812	3	3	3	3	2	3	0	0	0	0
	3 *P. chlororaphis* IFO 3506	3	3	3	3	2	3	2		0	
	4 *P. chlororaphis* IFO 3904	3	3	3	3	1	3	1		0	
	5 *P. chlororaphis* ATCC 17810	3	3	3	3	1	3	0	0	0	0
	6 *P. pyrrocinia* ATCC 15958	3	3	3	3	0		3	3	3	3
	7 *P. fluorescens* IFO 3507	3	3	3	3	0	2	2		2	
	8 *P. riboflavina* IFO 3140	2	3	2	3	2	3	2	3	2	3
	9 *P. convexa* IFO 3757	3	3	2	2	0	0	0	0	0	0
	10 *P. fragi* IFO 3458	3	3	3	3	3	2	2		0	
	11 *P. ovalis* IFO 3738	3	3	2	3	0	0	0	0	0	0
B *Enterobacteriaceae*	12 *Bacterium ketoglutaricum* B-4	3	3	0	0	0	0	0	0	0	0
	13 *Escherichia coli* mutaflor	3	3	0	0	0	0	0		0	
	14 *Citrobacter freundii* IRP-S-87	3	3	0	0	0	0	0	0	0	0
	15 *Aerobacter aerogenes* ATCC 8724	3	3	0	0	0	0	0		0	
	16 *Proteus mirabilis* OM-8	2	3	1	3	0		0		0	
	17 *Proteus vulgaris* YO-5	3	3	0	0	0	0	2		2	
C Asp-Gp bacteria	18 *Brevibacterium ammoniagenes* ATCC 6871	3	3	2	3	±	3	2	3	0	0
	19 *Brevibacterium linens* ATCC 9172	2	3	2	3	2	3	1	3	0	3
	20 *Corynebacterium glutamicum* ATCC 13057	3	3	3	3	2	2	3		0	
	21 *Micrococcus flavus* IFO 3242	3	3	3	3	3	3	2	3	0	0
	22 *Microbacterium flavum* ATCC 10340	3	3	3	3	3	3	2	3	0	0
	23 *Arthrobacter simplex* ATCC 6946	2	3	2	3	1	3	2	3	0	0
	24 *Corynebacterium equi* B-271-1	2	3	3	3	2	3	2	3	2	3
	25 *Mycobacterium phlei* ATCC 19249	2	3	2	3	2	3	1	2	±	2
D *Bacillus*	26 *B. cereus* IFO 3001	3	3	0	0	0	0	3	3	3	3
	27 *B. cereus* 60-6	3	3	0	0	0	0	3	3	3	3
	28 *B. subtilis* IFO 3007	3	3	0	0	0	0	2	3	±	2
	29 *B. subtilis* AR-30	3	3	0	0	0	0	3	3	0	0
	30 *B. pumilus* TL-47	3	3	0	2	0	0	3	3	3	3
	31 *B. licheniformis* ATCC 12199	3	3	±	3	0	±	2	3	3	3
	32 *B. circulans* NRRL B3313	3	3	2	3	0	0	3	3	3	3
	33 *B. laterosporus* 340-19	3	3	0	3	0	0	3	3	3	3
	34 *B. pulvifaciens* CB-57	3	3	1	2	0	0	3	3	3	3
	35 *B. brevis* Ak-4	3	3	0	0	0	0	1	3	1	3
	36 *B. polymyxa* AR-110	3	3	0	0	0	0	3	3	3	3
	37 *B. circulans* Bz-43	3	3	0	0	0	0	3	3	3	3
	38 *B. sphaericus* ATCC 7055	3	3	2	3	1	2	3	3	0	0

Table 1. (Continued)

		Control		Nalidixic acid				Colistin			
				10 μg/ml		50 μg/ml		5 μg/ml		25 μg/ml	
		4 days	10 days	4 days	10 days	4 days	10 days	4 days	10 days	4 days	10 days
E *Micromonospora*	39 *M. purpurea* NRRL 2953	2	3	2	3	±	2	1	2	0	0
	40 *M. halophytica* subsp. *nigra* NRRL 3097	3	3	3	3	3	3	±	2	0	0
	41 *M. echinospora* NRRL 2985	3	3	3	3	2	3	0	±	0	0
	42 *M. echinospora* subsp. *inyoensis* NRRL 3292	2	3	1	3	1	3	0	0	0	0
	43 *M. carbonacea* NRRL 2972	3	3	3	3	3	3	2	3	0	0
	44 *M. chalcea* subsp. *flavida* NRRL 3222	3	3	3	3	3	3	1	3	0	0
	45 *M. megalomicea* NRRL 3274	3	3	3	3	3	3	3	3	0	0
	46 *M. rosaria* NRRL 3718	3	3	2	3	2	3	3	3	0	0
	47 *M. grisea* NRRL 3800	3	3	3	3	2	3	0	0	0	0
	48 *M. rhodorangea* NRRL 5326	2	3	2	3	2	3	0	0	0	0
F *Streptomyces*	49 *S. griseus* NRRL 3851	3	3	3	3	3	3	3	3	0	0
	50 *S. lactamdurans* NRRL 3802	3	3	2	3	2	3	3	3	3	3
	51 *S. kanamyceticus* ATCC 12853	3	3	3	3	2	2	3	3	3	3
	52 *S. lincolnensis* ATCC 25499	3	3	3	3	3	3	3	3	0	0
	53 *S. tenebrarius* ATCC 17920	3	3	3	3	2	2	3	3	3	3
	54 *S. vinaceus* ATCC 25510	3	3	3	3	3	3	3	3	3	3
	55 *S. humidus* ATCC 12760	3	3	3	3	3	2	2	3	0	0
	56 *S. fradiae* Wacksmania 3535	3	3	2	2	2	2	2	3	2	2
	57 *S. ribosidificus* ATCC 21294	3	3	2	3	2	3	1	1	0	0
	58 *S. kasugaensis* ATCC 15714	2	3	2	3	2	3	2	3	2	3
	59 *S. levoris* ATCC 5876	3	3	3	3	3	3	3	3	0	0
	60 *S. erythreus* F-188	2	3	2	3	2	3	2	3	2	3
	61 *S. hygroscopicus* K-775	3	3	3	3	3	3	2	2	0	0
	62 *S. antibioticus* E-662	3	3	2	3	2	3	3	3	0	0
	63 *S. lavendulae* K-433	2	3	2	3	2	3	2	3	0	0

[a] Assay conditions: Agar dilution method was used in this experiment. About 5×10^5 CFU per ml of each cell suspension was inoculated onto Gly-IM agar which contained the indicated concentration of nalidixic acid or colistin. 3 signifies good growth, 2 moderate growth, 1 weak growth, ± very poor growth, and 0 no growth.

of other bacteria from soil samples were determined. Growth was measured by replicate plating using two antibiotic mixtures: Mixture A; colistin 5 μg/ml, nalidixic acid 10 μg/ml and cycloheximide 30 μg/ml. Mixture B; colistin 10 μg/ml, nalidixic acid 20 μg/ml and cycloheximide 30 μg/ml. As controls, an antibiotic-free agar medium (D) and a cycloheximide (30 μg/ml) containing agar medium (C) were also tested. The experimental conditions and the results of these tests are shown in Table 2.

Both of the test mixtures were effective in enriching for Asp-Gp bacteria over other Gram-positive and negative bacteria. The addition of 30 μg/ml of cycloheximide to the agar medium had no effect on bacterial growth; however, it was essential to prevent fungal contamination.[27] The Mixture B (colistin 10 μg/ml, nalidixic acid 20 μg/ml) was more selective than the Mixture A (colistin 5 μg/ml and nalidixic acid 10 μg/ml). It was especially useful for soil samples which contained many organisms other than

Table 2. Optimum concentrations of nalidixic acid and colistin for enrichment of Asp-Gp bacteria[a].

Mixture[b]	Growth[d]				Mixture[b]	Growth[d]			
	A	B	C	D		A	B	C	D
1 *P. aeruginosa* IFO 3449	0	0	5	5	10 *Mycob. phlei* ATCC 19249	3	3	3	3
2 *P. chlororaphis* IFO 3812	0	0	5	5	11 *B. cereus* IFO 3001	0	1[c]	5	5
3 *P. fluorescens* IFO 3507	±	0	5	5	12 *B. cereus* 60-6	0	0	5	5
4 *P. riboflavinus* IFO 3142	3	3	3	3	13 *B. pumilus* TL-47	2[c]	0	5	5
5 *A. aerogenes* ATCC 8724	0	0	5	5	14 *B. circulans* NRRL B3313	5	1[c]	5	5
6 *B. ammoniagenes* ATCC 6871	5	5	5	5	15 *B. sphaericus* ATCC 7055	5	2[c]	5	5
7 *Microcc. flavus* IFO 3242	5	5	5	5	16 *S. ribosidificus* ATCC 21294		0	5	5
8 *A. simplex* ATCC 6946	3[c]	2[c]	5	5	17 *S. hygroscopicus* K-775	1[c]	0	5	5
9 *Coryneb. equi* B-271-1	5	5	5	5	18 *S. lavendulae* K-433	3	0	5	5

a) Test conditions: Agar dilution method was used in this test. About 5×10^5 CFU per ml of each cell suspension was inoculated onto Gly-IM agar. The relative amount of growth for each strain was determined visually during 1~7 days incubation at 28°C.

b) Mixture A; 5 μg colistin, 10 μg nalidixic acid and 30 μg/ml of cycloheximide. Mixture B; 10 μg colistin, 20 μg nalidixic acid and 30 μg/ml of cycloheximide. Mixture C; 30 μg/ml of cycloheximide only. Mixture D; Control.

c) Very slow growth.

d) 5 signifies good growth, 3 moderate growth, 2 significant growth, 1 poor growth, ±very poor growth, and 0 no growth.

Asp-Gp bacteria. The Mixture A was less-selective than the B Mixture but was more safety for sorting out AsP-Gp bacteria. *Arthrobacter simplex* ATCC 6946 grew more slowly on the Mixture B agar than on the Mixture A. We chose the Mixture A for general use and the Mixture B for soil samples containing abundant amounts of contaminants.

Model Experiments

Using *Pseudomonas aeruginosa* IFO 3812, *Aerobacter aerogenes* ATCC 8724 and *Bacillus cereus* IFO 3001 as model contaminants, the enrichment effect of the Mixture A was tested for *Micrococcus flavus* IFO 3242. Three lots were prepared, each containing different concentrations of the contaminants. Test conditions and results are shown in Table 3.

Table 3. Model experiment of "Asp-GP-shifted" isolation[a].

		Lot I (CFU)	Lot II (CFU)	Lot III(CFU)
Bacteria added	1 *Micrococcus flavus* IFO 3242	45	45	45
	2 *Pseudomonas aeruginosa* IFO 3812	220	2200	22000
	3 *Aerobacter aerogenes* ATCC 8724	230	2300	23000
	4 *Bacillus cereus* IFO 3001	240	2400	24000
Bacteria colonized	1 *Micrococcus flavus* IFO 3242	45	50	50
	2 *Pseudomonas aeruginosa* IFO 3812	0	0	0
	3 *Aerobacter aerogenes* ATCC 8724	0	0	0
	4 *Bacillus cereus* IFO 3001	0	0	0

a) Gly-IM agar containing 5 μg/ml of colistin, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide. 28°C for 2 days.

Forty-five colony forming unit (CFU) of *Micrococcus flavus* IFO 3242 were plated on isolation medium containing the Mixture A of antibiotics along with 220 CFU of *P. aeruginosa* IFO 3812, 230 CFU of *Aerob. aerogenes* ATCC 8724 and 240 CFU of *B. cereus* IFO 3001 (Lot I). Similar platings were done using 10-fold (Lot II) and 100-fold (Lot III) numbers of the contaminants. As controls, the three mixtures of different bacteria (Lot I, II and III) were also plated on basal isolation agar medium.

No *Micrococcus flavus* colonies could be detected on antibiotic-free control plates. However, almost all *Micrococcus flavus* bacteria added were selectively isolated on the Mixture A agar plates. No contaminant colony was detected on the three lots of isolations.

From these results, it is clear that the Mixture A is useful in isolating an Asp-Gp bacterium from samples containing about 5 to 500-fold more Gram-positive and negative contaminants.

Trial Isolation of Asp-Gp Bacteria from 3 Soil Samples

A trial isolation was done for Asp-Gp bacteria from 3 soil samples collected from Osaka district. The Mixture A (see Table 2) was used for the isolation. The isolated strains were tentatively classified as described in Table 4 by Gram-staining, morphology at early and late stage, cell size, color of colony, and acid formation from glucose. The details of this trial and the results are shown in Table 4.

A total of 63 different colonies were isolated from the 3 soil samples. Of these, 16 strains were found to be contaminants (9 strains of *Streptomyces* and 7 strains of Gram-negative bacteria) while the remaining 47 strains were Asp-Gp bacteria. The 47 strains were grouped into 3 major groups, *i.e.*, *Arthrobacter* type, *Corynebacterium* type and *Micrococcus* type (see Table 4). These groups were subdivided into 3, 19 and 4 groups, respectively by tentative taxonomic checking. This means at least 26 kinds of Asp-Gp bacteria were obtained from 3 soil samples by the isolation method. From the same soil samples, only several kinds of the Asp-Gp bacteria were isolated by the standard dilution-plate method. This method proved to be more effective than the standard method for Asp-Gp bacteria isolation.

Discussion

Three different types of isolation methods have been used to isolate selected groups of bacteria from natural sources. The first type of isolation depends on the physical separation of the desired group of bacteria from other contaminants before plating. GLEDHILL and CASIDA[28,29] and SUTHERLAND[30~32] enriched catalase-negative soil bacteria, which included *Agromyces ramosus* and microcyst-forming bacteria such as *Myxobacteria* and *Sporocytophaga*, by ultrasonication of the source samples. HALL, HOGG and PHILLIPS[33] separated *Salmonella typhimurium* and *E. coli* strains by gradient elution from a DEAE-cellulose column, and FORSHAW[34] separated *Mycoplasma* strains by electrophoresis. LACEY and DUTVIEWICZ[35] enriched *Streptomyces* in moldy hay by use of a sedimentation chamber, and TRESNER and HAYES[36] used a replicate printing using polyurethane foam to isolate varieties of microorganisms from soil samples. This type of isolation, is however, difficult for soil bacteria because the bacteria adsorbed tightly onto soil particles.[30~32]

The second type of isolation is to colonize a particular group of bacteria under controlled conditions for their growth, *i.e.* nutrients, temperature, oxygen and pH. Selective isolation of *Azotobacter, Rhizobium*, and halophilic or sulfur bacteria[1] depends on this type of isolation. Many techniques of this type have been reported for various kinds of bacteria.[1,10]

The third type of isolation is the use of inhibitors to depress the growth of other contaminating bacteria. This type of isolation is the most popular and widely used technique for the selective isolation of a group of bacteria. Various chemicals and antibiotics have been used for this purpose. SANDS and ROVIRA[11] isolated fluorescent *Pseudomonads* by using novobiocin, penicillin and cycloheximide, while

Table 4. The Asp-Gp bacteria obtained from 3 soil samples by "Asp-Gp-shifted" isolation.

	Morphology[a] cell-shape		Cell-width	Gram-staining[b]		Color of colony[c]	Acid from glucose[d]	Strains[e]
	Early	Late	(μm)	E	L			
A *Arthrobacter*-type								
Group 1	Rod	Coccoid	>0.5	Gn	Gp	None	−	A-16
Group 2	Rod	Coccoid	≥0.5	Gn	Gp	Cream-cray	+	B-1
Group 3	Rod	Rod	<0.5	Gn	Gp	None	−	B-7
B *Corynebacterium*-type								
Group 1	Rod	Coccoid	>0.5	Gp	Gp, Gn	Cream	+	A-17, -20
Group 2	Rod	Rod & Coccoid	>0.5	Gp	Gp, Gn	Citron	±	C-6
Group 3	Rod	Rod & Coccoid	>0.5	Gp	Gp, Gn	Yellow	−	B-19, -18
Group 4	Rod	Rod & Coccoid	≑0.5	Gp	Gp, Gn	Citron	−	A-1, -2, -3
Group 5	Rod	Rod & Coccoid	≑0.5	Gp	Gp, Gn	Cream-yellow	−	B-15, -16
Group 6	Rod	Coccoid	>0.5	Gp	Gp	None	+	A-4, -7
Group 7	Rod	Coccoid	>0.5	Gp	Gp	None	±	B-17
Group 8	Rod	Coccoid	>0.5	Gp	Gp	None	−	C-19
Group 9	Rod	Coccoid	>0.5	Gp	Gp	Citron	±	A-8
Group 10	Rod	Coccoid	>0.5	Gp	Gp	Cream-gray	−	C-2, -4, -14
Group 11	Rod	Coccoid	>0.5	Gp	Gp	Citron-white	±	C-16
Group 12	Rod	Coccoid	>0.5	Gp	Gp	Yellow-translucent	−	C-5, -17
Group 13	Rod	Rod & Coccoid	>0.5	Gp	Gp	None	−	B-5, -6, -10 C-1, -8
Group 14	Rod	Coccoid	>0.5	Gp	Gp	Yellow-translucent	+	A-5, -6, -9
Group 15	Rod	Coccoid	≑0.5	Gp	Gp	None	±	B-3, -4, -8
Group 16	Rod	Coccoid	<0.5	Gp	Gp	Whitish-citron	+	A-21
Group 17	Rod	Rod	<0.5	Gp	Gp	Citron	+	A-23
Group 18	Rod	Rod & Coccoid	<0.5	Gp	Gp	Orange	+	A-14, -15, -19
Group 19	Rod	Rod	<0.5	Gp	Gp	Lavender	−	A-18
C *Micrococcus*-type								
Group 1	Sphere	Sphere	>0.5	Gp	Gp	Yellow	−	C-3, -15
Group 2	Sphere	Sphere	>0.5	Gp	Gp	Whitish yellow	−	C-7, -9
Group 3	Sphere	Sphere	>0.5	Gp	Gp	Cream-yellow	−	C-12, -18
Group 4	Sphere	Sphere	>0.5	Gp	Gp	Pink	−	C-10, A-10

a) Morphological checks of each strains were done after incubation at 28°C for 14~18 hours, 36~48 hours, 86~96 hours and 158~170 hours. "Early" means after 14~18 hours and "Late", the results after 2 to 7 days of incubation. Cell width (in μm) was measured at 1 to 2 days of incubation.

b) Gram-staining of each strains was done at the same time of incubation as the morphological observation. Gp; Gram-positive and Gn; Gram-negative.

c) Color of colony was determined at 2~3 days of incubation.

d) Acid formation from glucose was tested by the following agar medium at 28°C for 1 to 7 days: 1.0% glucose, 0.5% peptone, 0.3% yeast extract, 0.5% NaCl, 0.0008% bromcresol purple, 1.25% agar, pH 7.0.

e) Strain number from soil A, B and C.

ORCHARD and GOODFELLOW[22] enriched *Nocardia* in soil samples by using chlortetracycline, cycloheximide and nystatin. PARK, RAYMAN and STANKIEWICZ[14] separated *Shigella* in the presence of *E. coli* by the use of 4-chloro-2-cyclopentyl-β-galactopyranoside. For Asp-Gp bacteria, MILLER and NEVILLE[25] reported recently a medium which was satisfactory for selective isolation of *Arthrobacter* spp. from a variety of soils.

This isolation technique has made possible the isolation of Asp-Gp bacteria for antibiotic screening as efficient as the isolation of *Streptomyces, Micromonospora, Pseudomonas*, and *Bacillus*. Nalidixic acid at 10 to 20 μg/ml concentrations was used in combination with 5 to 10 μg/ml of colistin in this isolation. It has been known that nalidixic acid is effective against enteric bacteria[37] but its broad activity against *Bacillus* species has not been reported previously.

A hygromycin and epihygromycin producer, *Corynebacterium equi* No. 2841,[8] was isolated from among 3,900 isolates of Asp-Gp bacteria by this procedure.

References

1) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY's Manual of Determinative Bacteriology. 8th *ed.* The Williams & Wilkins Co., Baltimore, 1974
2) UMEZAWA, H.: Index of antibiotics from Actinomycetes. pp. 124~126, The University of Tokyo Press, Tokyo, 1967
3) KONISHI, M.; M. KIMEDA, H. TSUKIURA, T. YAMAMOTO, T. HOSHIYA, T. MIYAKI, K. FUJISAWA, H. KOSHIYAMA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. I. Production, isolation and properties. J. Antibiotics 26: 752~756, 1973
4) KONISHI, M.; M. NARUISHI, T. TSUNO, H. TSUKIURA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. II. Structure of oxamicetin. J. Antibiotics 26: 757~764, 1973
5) TOMITA, K.; Y. UENOYAMA, K. FUJISAWA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. III. Taxonomy of the oxamicetin-producing organism. J. Antibiotics 26: 765~770, 1973
6) SUZUKI, T.; H. HONDA & R. KATSUMATA: Production of antibacterial compounds analogous to chloramphenicol by *n*-paraffin grown bacterium. Agr. Biol. Chem. 36: 2229~2232, 1972
7) FRENCH, J. C.; S. T. SHORSE, J. D. HOUDIS, G. P. WOOD & I. F. ANDERSON: Erythromycin process. U.S. Patent 3,551,294, 1970
8) WAKISAKA, Y.; K. KOIZUMI, Y. NISHIMOTO, M. KOBAYASHI & N. TSUJI: Hygromycin and epihygromycin from a bacterium, *Corynebacterium equi* No. 2841. J. Antibiotics 33: 695~704, 1980
9) LEWIN, R. A. & D. M. LOUNSBERY: Isolation, cultivation and characterization of Flexibacteria. J. Gen. Microbiol. 58: 145~170, 1969
10) BLAIR, L. E.; E. H. LENNETTE & J. P. TRUANT (*ed.*): Manual of Clinical Microbiology. pp. 649, 654 & 650 and pp. 265~279, American Society for Microbiology, 1970
11) SANDS, D. C. & A. D. ROVIRA: Isolation of fluorescent *Pseudomonads* with a selective medium. Appl. Microbiol. 20: 513~514, 1970
12) CHRISTENSEN, P. J. & F. D. COOK: The isolation and enumeration of cytophages. Can. J. Microbiol. 18: 1933~1940, 1972
13) SUTHERLAND, L. W.: Ultrasonication: An enrichment technique for microcyst-forming bacteria. J. Appl. Bacteriol. 41: 185~188, 1976
14) PARK, C. E.; M. K. RAYMAN & Z. K. STANKIEWICZ: Improved procedure for selective enrichment of *Shigella* in the presence of *E. coli* by use of 4-chloro-2-cyclopentyl-β-galactopyranoside. Can. J. Microbiol. 23: 563~566, 1977
15) MAROLD, L. M.; R. FREEDMAN, R. E. CHAMBERLAIN & A. VALDIVIESO-GARCIA: New selective agent for isolation of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 41: 977~980, 1981
16) LINGAPPA, Y. & J. L. LOCKWOOD: A chitin medium for isolation, growth and maintenance of *Actinomycetes*. Nature 189: 158~159, 1961
17) EL KNKEEB, M. A. & H. A. LECHEVALIER: Selective isolation of aerobic *Actinomycetes*. Appl. Microbiol. 11: 75~77, 1963
18) AGATE, A. D. & J. V. BHAT: A method for the preferential isolation of *Actinomycetes*. Antonie Van Leewenhoef 27: 297, 1963
19) KUSTER, E. & S. T. WILLIAM: Selection of media for isolation of *Streptomycetes*. Nature 202: 928, 1964
20) NONOMURA, H. & Y. OHARA: Distribution of *Actinomycetes* in soil. A cellulose method effect for both

preferential isolation and enumeration of *Micromonospora* and *Streptosporangium* strains in soil. II. Classification of the isolates. J. Ferment. Technol. 47: 701~709, 1969

21) LAVROVA, N. V.; T. P. PREBRAZHENSKAYA & M. A. SVENSHNIKOVA: Isolation of soil *Actinomycetes* on selective media with rubromycin. Antibiotiki 17: 965~970, 1972

22) ORCHARD, V. A. & M. GOODFELLOW: The isolation of *Nocardia* from soil using antibiotics. J. Gen. Microbiol. 85: 160~162, 1974

23) CHROMONOVA, N. T.: Isolation of *Actinomadulae* from soils on selective media with kanamycin and rifampicin. Antibiotiki 23: 22~26, 1978

24) IVANITSKAYA, L. P.; S. M. SINGAL, M. V. BIBIKOVA & S. M. VOTIROU: Direct isolation of cultures of the genus *Micromonospora* on selective media with gentamicins. Antibiotiki 23: 690~692, 1978

25) MILLER, K. W. & M. E. NEVILLE: Media for isolation of *Arthrobacter* spp. from soil. Ann. Meeting of American Society Microbiol. 77: 230, 1977

26) ARIMA, K.; H. IMANAGA, M. KOUSAKA, A. FUKUTA & G. TAMURA: Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. Agr. Biol. Chem. 28: 575~576, 1964

27) CORBAZ, R.; P. H. GREGORY & M. C. LACEY: Thermophilic and mesophilic *Actinomycetes* in mouldy hay. J. Gen. Microbiol. 32: 449~455, 1963

28) CASIDA, L. E.: Abundant microorganism in soil. Appl. Microbiol. 13: 327~334, 1965

29) GLEDHILL, W. E. & L. E. CASIDA, Jr.: Predominant catalase-negative soil bacteria. III. Agromyces, gen. nov., microorganisms intermediary to *Actinomyces* and *Nocardia*. Appl. Microbiol. 18: 340~349, 1969

30) MULLER, G. & B. HICKISCH: The adsorption of soil bacteria on substrates with special reference to secondary clay minerals and ionic exchangers on the basis of artificial resin. Zent. Bakteriol., Parasit., Infektion. Hygiene 125: 333~362, 1970

31) MULLER, G. & B. HICKISCH: On the adsorption behaviour of bacteria in the soil. Symp. Biol. Hung. 11: 263~269, 1972.

32) MEADOWS, P. S.: The attachment of bacteria on solid surface. Arch. Mikrobiol. 75: 347~381, 1971

33) HALL, A. N.; S. D. HOGG & G. O. PHILLIPS: Gradient elution of *Salmonella typhimurium* and *Escherichia coli* strains from a DEAE-cellulose column. J. Appl. Bacteriol. 41: 189~192, 1976

34) FORSHAW, K. A.: Electrophoretic patterns of strains of *Mycoplasma pulmonis*. J. Gen. Microbiol. 72: 493~500, 1972

35) LACEY, J. & J. DUTVIEWICZ: Isolation of *Actinomycetes* and fungi from mouldy hay using a sedimentation chamber. J. Appl. Bacteriol. 41: 315~319, 1976

36) TRESNER, H. D. & J. A. HAYES: Improved methodology for isolating soil microorganisms. Appl. Microbiol. 19: 186~187, 1970

37) WEINER, R. M.; M. J. VOLL & T. M. COOK: Nalidixic acid for enrichment of auxotrophs in cultures of *Salmonella typhimurium*. Appl. Microbiol. 28: 579~588, 1974