A SELECTIVE ISOLATION PROCEDURE FOR *PSEUDOMONAS* BACTERIA

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A selective isolation medium was devised for *Pseudomonas* bacteria. An antibiotic mixture which contained 10 μg per ml of cerexin A, 10 μg per ml of nalidixic acid and 30 μg per ml of cycloheximide was used. With this antibiotic medium, 58 strains of bacteria presumed to be *Pseudomonas* which were subdivided into 18 taxonomically different groups were isolated from 3 soil samples with 9% of contaminants. With this method, it was possible to isolate a *Pseudomonas* bacterium from a sample containing about 400 times as many other Gram-positive and -negative bacteria.

Pseudomonas bacteria have been recognized as sources of new antibiotics. Pyrrolnitrin,[1] sorbistin,[2] as well as sulfazecin and isosulfazecin[3] have been obtained from this group of bacteria.

SANDS and ROVIRA[4] have reported a selective medium for fluorescent *Pseudomonas* which containes novobiocin, penicillin and cycloheximide, and MAROLD *et al.*[5] have found a new selective agent, 9-chloro-9-(-4-diethylaminophenyl)-10-phenylacridan, for the isolation of *P. aeruginosa*. Deoxycholate agar,[6] MACCONKEY agar containing bile acid,[6] and eosine-methylene blue agar[6] are also used for clinical isolation of *Pseudomonas* bacteria.

In this study, we have developed a more efficient procedure utilizing cerexin A and nalidixic acid for isolation of *Pseudomonas* bacteria. Cerexin A, a peptidic antibiotic from *Bacillus cereus* 60–6,[7] is only active against Gram-positive bacteria. Nalidixic acid is a well-known inhibitor of enteric Gram-negative bacteria[8] and *Bacillus* strains.[9] Addition of nalidixic acid at appropriate concentrations to the isolation medium was however expected to be useful for selecting *Pseudomonas* bacteria among various enteric Gram-negative bacteria. The combination of cerexin A and nalidixic acid was expected to be effective for selecting *Pseudomonas* bacteria also among various Gram-positive and -negative bacteria.

Materials and Methods

Bacteria Used

Sixty-three strains of Gram-positive and -negative bacteria including *Streptomyces* and *Micromonospora*, (see Table 1) were subjected to sensitivity tests against cerexin A and nalidixic acid. 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 Collections (ATCC). The others were isolated from various soil samples in this laboratory.

Sensitivity Test

An agar dilution method was used to determine the sensitivities of various Gram-positive and -negative bacteria against cerexin A and nalidixic acid. About one μl of a $10^5 \sim 10^6$ colony forming units (CFU) per ml suspension was inoculated onto Gly-IM agar medium (see below) which contained variable concentrations of cerexin A or nalidixic acid. The inoculated plates were incubated at 28°C for 1 to 10 days, and the growth of each inoculate at 2, 4, 6, and 10 days stage was measured by naked eye.

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

		Control		Nalidixic acid				Cerexin A			
				10 μg/ml		50 μg/ml		25 μg/ml		100 μg/ml	
	Days	2	6	2	6	2	6	2	6	2	6
A	*Pseudomonas*										
1	*P. aeruginosa* IFO 3449	3	3	2	3	0	3	3	3	3	3
2	*P. aeruginosa* IFO 3812	3	3	3	3	2	3	2	3	1	3
3	*P. chlororaphis* IFO 3506	3	3	3	3	2	3	3	3	3	3
4	*P. chlororaphis* IFO 3904	3	3	3	3	1	3	3	3	3	3
5	*P. chlororaphis* ATCC 17810	3	3	3	3	1	3	3	3	3	3
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	3	3	3	3
8	*P. riboflavinus* IFO 3140	2	3	2	3	2	3	0	0	0	0
9	*P. syncyanea* IFO 3757	3	3	2	2	0	0	3	3	2	3
10	*P. fragi* IFO 3458	3	3	3	3	3	2	3	3	3	3
11	*P. putida* IFO 3738	3	3	2	3	0	0	3	3		
B	*Enterobacteriaceae*										
12	*Bacterium ketoglutaricum* B-4	3	3	0	0	0	0	3	3	3	3
13	*Escherichia coli* mutaflor	3	3	0	0	0	0	3		3	
14	*Citrobacter freundii* IRR-S-87	3	3	0	0	0	0	3	3	3	3
15	*Aerobacter aerogenes* ATCC 8724	3	3	0	0	0	0	3		3	
16	*Proteus mirabilis* OM-8	2	3	1	3	0	0	2	3	2	3
17	*Proteus vulgaris* YO-5	3	3	0	0	0	0	3		3	
C	Asporogenous Gram-positive bacteria										
18	*Brevibacterium ammoniagenes* ATCC 6871	3	3	2	3	±	3	0	0	0	0
19	*Brevibacterium linens* ATCC 9172	2	3	2	3	2	3	0	0	0	0
20	*Corynebacterium glutamicum* ATCC 13057	3	3	3	3	2	2	0	0	0	0
21	*Micrococcus luteus* ATCC 10240	3	3	3	3	3	3	0	0	0	0
22	*Microbacterium flavum* ATCC 10340	3	3	3	3	3	3	0	0	0	0
23	*Arthrobacter simplex* ATCC 6946	3	3	3	3	3	3	0	0	0	0
24	*Corynebacterium equi* B-271-1	2	3	2	3	1	3	0	0	0	0
25	*Mycobacterium phlei* ATCC 19249	3	3	2	3	2	3	0	0	0	0
D	*Bacillus*										
26	*B. cereus* IFO 3001	3	3	0	0	0	0	0	0	0	0
27	*B. cereus* 60–6	3	3	0	0	0	0	3	3	0	0
28	*B. subtilis* IFO 3003	3	3	0	0	0	0	0	0	0	0
29	*B. subtilis* AR-30	3	3	0	0	0	0	0	0	0	0
30	*B. pumilus* TL-47	3	3	0	2	0	0	0	0	0	0
31	*B. licheniformis* ATCC 12199	3	3	±	3	0	±	0	0	0	0
32	*B. circulans* NRRL B3313	3	3	2	3	0	0	0	0	0	0
33	*B. laterosporus* 340–19	3	3	0	3	0	0	0	0	0	0
34	*B. pulvifaciens* CB-57	3	3	1	2	0	0	0	0	0	0
35	*B. brevis* Ak-4	3	3	0	0	0	0	0	0	0	0
36	*B. polymyxa* AR-110	3	3	0	0	0	0	0	0	0	0
37	*B. circulans* Bz-43	3	3	0	0	0	0	0	0	0	0
38	*B. sphericus* ATCC 7055	3	3	3	3	1	2	0	0	0	0

Table 1. (continued)

		Control		Nalidixic acid				Cerexin A			
				10 μg/ml		50 μg/ml		25 μg/ml		100 μg/ml	
	Days	4	10	4	10	4	10	4	10	4	10
E	*Micromonospora*										
39	*M. purpurea* NRRL 2953	2	3	2	3	±	2	0	±	0	±
40	*M. halophytica* subsp. *niger* NRRL 3097	3	3	3	3	3	3	0	0	0	0
41	*M. echinospora* NRRL 2985	3	3	3	3	2	3	0	0	0	0
42	*M. echinospora* subsp. *inyoensis* NRRL 3292	2	3	1	3	1	3	2	2	2	2
43	*M. carbonacea* NRRL 2972	3	3	3	3	3	3	0	0	0	0
44	*M. chalcea* subsp. *flavida* NRRL 3222	3	3	3	3	3	3	0		0	0
45	*M. megalomicea* NRRL 3272	3	3	3	3	3	3	0	0	0	0
46	*M. rosaria* NRRL 3718	3	3	2	3	2	3	0	0	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	0	0	0	0
50	*S. lactamdurans* NRRL 3802	2	3	2	3	1	3	0	0	0	0
51	*S. kanamyceticus* ATCC 12853	3	3	3	3	2	2	0	0	0	0
52	*S. lincolnensis* ATCC 25466	2	3	2	3	2	3	0	0	0	0
53	*S. tenebrarius* ATCC 17920	2	3	2	3	1	2	0	0	0	0
54	*S. vinaceus* ATCC 25510	3	3	3	3	2	3	0	0	0	0
55	*S. humidus* ATCC 12760	3	3	3	3	2	3	0	0	0	0
56	*S. fradiae* Waksman 3535	2	3	2	2	2	2	0	0	0	0
57	*S. ribosidificus* ATCC 21294	2	3	2	3	2	2	0	0	0	0
58	*S. kasugaensis* ATCC 15714	2	3	2	3	2	3	1	3	0	0
59	*S. erythraeus* F-188	3	3	3	3	3	3	0	0	0	0
60	*S. hygroscopicus* K-775	2	3	2	3	±	3	0	0	0	0
61	*S. antibioticus* E-662	2	3	2	3	2	3	0	0	0	0
62	*S. lavendulae* K-433	2	3	2	3	1	3	0	0	0	0
63	*S. levoris* ATCC 5876	3	3	3	3	3	3	0	0	0	0

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.

Selective Isolation Procedure for *Pseudomonas*

About 0.5 g of a fresh soil sample was suspended in 5 ml of sterile saline solution containing 0.01% w/v $MgSO_4 \cdot 7H_2O$ and stirred vigorously with two or three glass beads (about 4 mm in diameter) in 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 diluted 10-fold serially to $10^{-2} \sim 10^{-5}$ and 0.5 ml of each dilution was plated onto 15 ml of Gly-IM agar medium (see below) which contained 10 μg/ml of cerexin A, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide. The inoculated agar plates were incubated at 28°C for 2 to 7 days for the *Pseudomonas* colonies to develop. All antibiotics used were sterilized by filtration. The 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% w/v Bacto-agar (Difco), pH 6.8.

Results and Discussion

Sensitivity Tests of Various Gram-positive and -negative Bacteria to Cerexin A and Nalidixic Acid

As shown in Table 1, 63 strains of various Gram-positive and -negative bacteria were tested for their sensitivities to cerexin A and nalidixic acid by an agar-dilution method (see Materials and Methods). The test bacteria were selected randomly from species of *Pseudomonas, Enterobacteriaceae*, asporogenous Gram-positive aerobes, *Bacillus, Micromonospora* and *Streptomyces*.

All *Pseudomonas* and *Enterobacteriaceae* strains tested grew normally on agar plates containing 25 or 100 μg/ml of cerexin A, except *P. riboflavinus* IFO 3140. In contrast, all strains of *Bacillus*, asporogenous Gram-positive aerobes, *Streptomyces* and *Micromonospora* were susceptible to the antibiotic at 25 and 100 μg per ml concentrations, with two exceptions, *B. cereus* 60–6 (a cerexin A producer,[7] was sensitive at 100 μg/ml) and *M. echinospora* subsp. *inyoensis* NRRL 3292 (a sisomicin producer,[10] grew slowly at 25 and 100 μg/ml). Cerexin A is therefore a useful inhibitor for Gram-positive bacteria. Sensitivity tests with these Gram-positive and -negative bacteria to novobiocin and penicillin were also done, but several of them were resistant to these antibiotics (data not shown).

All *Pseudomonas* strains tested grew well on agar plates containing 10 μg/ml of nalidixic acid. However, some were susceptible to 50 μg/ml of the antibiotic. The enteric Gram-negative bacteria tested were susceptible to 10 and 50 μg/ml of nalidixic acid, with one exception, *Proteus mirabilis* OM-8. Most of the *Bacillus* strains including *B. cereus* 60–6 (a cerexin A producer) were also sensitive to 10 μg/ml of nalidixic acid. The *Streptomyces* and *Micromonospora* strains tested were not inhibited by this antibiotic.

As shown in a previous study,[9] 30 μg/ml of cycloheximide inhibited most of fungal and yeast growth but had no effect on the growth of these test bacteria.

The above results clearly indicate that *Pseudomonas* bacteria could be selectively isolated by adding 25 μg/ml of cerexin A, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide into the isolation medium.

Optimum Concentrations of Cerexin A and Nalidixic Acid

With 22 strains of Gram-positive and -negative bacteria (see Table 2), the optimum concentrations of cerexin A and nalidixic acid needed to isolate *Pseudomonas* bacteria and minimize the number of other bacterial colonies from soil samples were determined. Growth was measured by replicate plating using two antibiotic mixtures: Mixture A; 10 μg/ml cerexin A, 10 μg/ml nalidixic acid and 30 μg/ml cycloheximide, and Mixture B; 10 μg/ml cerexin A, 20 μg/ml nalidixic acid and 30 μg/ml cycloheximide (see Table 2). The experimental conditions and the results of these tests are reported in Table 2.

The nine strains of *Pseudomonas* tested all grew well on both Mixture A and B agar plates. All other Gram-positive and -negative bacteria (see Table 2, 13 strains) did not grow on these agar mixtures. Therefore, both Mixture A and B could be usable for selective isolation of *Pseudomonas* bacteria. *P. syncyanea* IFO 3757, *P. fragi* IFO 3458 and *P. putida* IFO 3738, however, grew more slowly on Mixture B agar than on A. Therefore, we chose the Mixture A for routine isolation of *Pseudomonas*.

Model Experiments with the Selective Isolation Procedure for *Pseudomonas*

Using the Mixture A agar medium, selective isolation of *P. chlororaphis* ATCC 17810 was attempted from mixtures of three other bacteria, *Aerobacter aerogenes* ATCC 8724, *Micrococcus luteus* ATCC 10240 and *B. cereus* 60–6, at various concentrations. The experimental conditions and results are shown

Table 2. Optimum concentrations of nalidixic acid and cerexin A for selective isolation of *Pseudomonas* strains[a].

	Growth on[d]				Growth on[d]		
	Control	Mixture A[b]	Mixture B[b]		Control	Mixture A[b]	Mixture B[b]
1 *P. aeruginosa* IFO 3449	5	5	4	13 *Br. ammoniagenes* ATCC 6871	5	0	0
2 *P. aeruginosa* IFO 3812	5	5	4	14 *Microc. luteus* ATCC 10240	5	0	0
3 *P. chlororaphis* IFO 3506	5	5	5	15 *Mycob. phlei* ATCC 19249	3	0	0
4 *P. chlororaphis* ATCC 17810	5	5	5	16 *B. cereus* 60–6	5	0	0
				17 *B. pumilus* TL-47	5	0	0
5 *P. fluorescens* IFO 3507	5	5	5	18 *B. laterosporus* 340–19	5	0	0
6 *P. riboflavinus* IFO 3140	3	4	4	19 *B. sphericus* ATCC 7055	5	0	0
7 *P. convexa* IFO 3757	5	5	3[c]	20 *Str. hygroscopicus* K-775	5	0	0
8 *P. fragi* IFO 3458	5	5	3[c]				
9 *P. ovalis* IFO 3738	5	5	3[c]	21 *Str. lavendulae* K-433	5	0	0
10 *Bact. ketoglutaricum* B-4	5	0	0	22 *Str. antibioticus* E-662	5	0	0
11 *E. coli* mutaflor	5	0	0				
12 *Ae. aerogenes* ATCC 8724	5	0	0				

[a] Test conditions. See "Materials and Methods". Incubated at 28°C for 1 to 7 days.
[b] Mixture A; 10 μg/ml of cerexin A, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide. Mixture B; 10 μg/ml of cerexin A, 20 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide.
[c] Slow growth.
[d] 5 signifies good growth, 4 fairly good growth, 3 fairly weak growth, and 0 no growth.

Table 3. Model experiments of the selective isolation procedure for *Pseudomonas*[a].

	Series I	Series II	Series III
Bacteria inoculated:			
1 *P. chlororaphis* ATCC 17810	60 CFU	60 CFU	60 CFU
2 *Aerob. aerogenes* ATCC 8724	230 CFU	2300 CFU	23000 CFU
3 *Micrococcus luteus* ATCC 10240	220 CFU	2200 CFU	22000 CFU
4 *Bacillus cereus* 60–6	200 CFU	2000 CFU	20000 CFU
Bacteria found:			
1 *P. chlororaphis* ATCC 17810	62 CFU	55 CFU	82 CFU
2 *Aerob. aerogenes* ATCC 8724	0 CFU	0 CFU	0 CFU
3 *Micrococcus luteus* ATCC 10240	0 CFU	0 CFU	0 CFU
4 *Bacillus cereus* 60–6	0 CFU	0 CFU	0 CFU

[a] Gly-IM agar containing 10 μg/ml of cerexin A, 10 μg/ml of nalidixic acid and 30 μg/ml of cycloheximide. 28°C for 2 days.

in Table 3.

In one test series, Series I in Table 3, 60 CFU of *P. chlororaphis* was plated on the Mixture A agar with 230 CFU of *A. aerogenes*, 220 CFU of *M. luteus* and 200 CFU of *B. cereus* (see Table 3). Similarly, 10- (Series II) or 100-fold (Series III) numbers of contaminants were also plated with 60 CFU of *P.*

Table 4. Bacteria presumed to be *Pseudomonas*, obtained from 3 soil samples by the selective isolation procedure[a].

Group	Cell width μm	Flagella[b]	Color of colony	Growth at 42°C[c]	Gelatin liquefy[d]	Acid from glucose[e]	Growth characteristics	Strains
1	<0.5	P–1	Yellow	−	−	+	Vicid structure	B-2, -17
2	<0.5	P–1	Orange	−	+	−		C-7, -25
3	>0.5	P–1	Lavender	−	+	−	Swollen shape	A-6
4	0.5	P–1	Pinkish gray	−	+++	−	Diffusible pigment	C-15
5	0.5	P≥1	None	−	−	+		B-1, -4, -5, -6
6	0.5	P>1	None	−	−	+		C-1, -2, -12, -13, -23
7	0.5	P–1	None	+	−	+		C-4, -5, -8, -18, -24
8	0.5	P≥1	None	+	−	+		A-3, -8, -11
9	>0.5	P>1	None	+	−	+	Swollen shape	C-3, -6, -9, -28
10	0.5	P>1	None	++	−	+		A-5
11	0.5	P≥1	None	−	−	±		C-16, -20, -21, -22
12	0.5	P≥1	None	−	−	±		A-1, -9, -17
13	<0.5	P–1	None	−	+++	−		B-13
14	>0.5	P–1	None	−	−	−		A-14, -15
15	0.5	P–1	None	+	−	−		C-10, -11, -14, -19, -26, -27
16	0.5	P–1	None	++	−	−		B-3, -8, -11, -19
17	0.5	P–1	None	++	−	±		B-7, -12, -14, -16
18	0.5	P–1	None	+	−	−		B-18
19	>0.5	Peri	Yellow	−	−	−	Translucent	A-12, -13, -16
20	0.5	Peri	None	−	−	±		A-2, -4

[a] Isolation conditions: Same as Table 3.
[b] P–1 signifies single polar flagellum, P≥1 mixture of single and oligopolar flagella, P>1 multipolar flagella, and Peri peritrichous flagella.
[c] ++ signifies good growth, +significant growth, and − no growth
[d] +++ signifies rapid liquefaction, + weak liquefaction, and − no liquefaction
[e] + signifies clear acid formation, ± weak acid formation, and − no acid formation.

chlororaphis (see Table 3).

All of the *P. chlororaphis* bacteria added were detected on the agar plates in every series of tests as distinct colonies. No colonies of the three contaminants were detected in any series of tests (see Table 3). This indicates that the Mixture A is useful for the selective isolation of *Pseudomonas* bacterium from samples containing about 400 times as many of Gram-positive or -negative contaminants.

Attempts at Isolation of *Pseudomonas* Bacteria from Three Soil Samples

Isolation of *Pseudomonas* bacteria was attempted from three soil samples by use of Mixture A. The soil samples were collected from Osaka district. The isolated strains were tentatively grouped by Gram-staining, OF-test, cell size, type of flagella, color of colony, growth at 42°C, gelatine liquefaction, acid formation from glucose and growth characteristics (see Table 4). The experimental conditions and results are described in Table 4.

A total of 63 strains were isolated from the three soil samples by this method. Among them, four

strains were Gram-positive bacteria, one strain was a yeast. The remaining 58 strains were Gram-negative aerobes, and they were taxonomically divided into 20 subgroups as shown in Table 4. Only two of those subgroups (5 strains) had peritrichous flagella, while the remaining 18 subgroups (53 strains) had polar flagellum or flagella.

Thus, at least 18 strains likely to be *Pseudomonas* were obtained from the three soil samples by this method. Only 9 of these 18 subgroups were obtained from the same soil samples by the standard dilution-plate method (data not shown). The method now presented, therefore, proved to be more efficient than the standard method for *Pseudomonas*-isolation.

Secondary selection of the isolates by this method was convenient for sorting out *P. aeruginosa* strains. Most of the *P. aeruginosa* strains were detected by growth test at 42°C and tests for pigment-formation on an appropriate agar medium.

This improved method should be applicable for isolation of *Pseudomonas* clinically and from the environment with slight modifications (temperature, medium composition). It can also be used to count *Pseudomonas* bacteria among various Gram-positive and -negative bacteria.

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