AN ENRICHMENT ISOLATION PROCEDURE FOR MINOR *BACILLUS* POPULATIONS

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In general, spores of *B. cereus*, *B. megaterium*, *B. sphaericus* and *B. subtilis* strains germinated uniformly within a short time of incubation in a germination medium. In contrast, spores of *B. circulans*, *B. brevis*, *B. laterosporus*, *B. pulvifaciens*, *B. polymyxa*, *B. pumilus*, *B. licheniformis* and *B. coagulans* strains were usually slow and/or uneven germinators under the same conditions of incubation.

The former group of *Bacillus* strains occur frequently in soils as the predominant population and the latter group of *Bacillus* species are found in many cases as minor populations. The minor populations of *Bacillus* were isolated with difficulty by the standard dilution-plate technique, but could easily be enriched by treating the soil sample in a germination medium for 2 to 3 hours at 30 to 35°C, followed by heating it at 65°C for 10 minutes ("minor-shifted isolation").

Using this technique, the minor *Bacillus* strains could be isolated from samples containing 100- to 1,000-fold more of the rapid germinators of *Bacillus*.

When screening for new antibiotics, the isolation procedure for antibiotic-producing microorganisms is one of the most critical steps in the screening.

Most saprophytic *Bacillus* bacteria can be easily selected and isolated from soil samples since their dormant spores germinate and grow on an appropriate nutrient medium after heat treatment which pasteurizes most of other bacteria in the samples. For glucose-retardatory *Bacillus* such as *B. firmus* and *B. lentus*, no sugar-containing media have been used.[1] *Bacillus macerans* strains can be efficiently isolated by incorporating 2-phenylacetic acid and fructose into the isolation medium as spore germinants.[2] Alkalophilic *Bacillus* such as *B. pasteurii* can be obtained by adding urea to the isolation medium.[1] Psychrophilic or thermophilic *Bacillus* such as *B. psychrophilus* and *B. macquariensis* or *B. stearothermophilus* and *B. coagulans* can be obtained by a low (below 10°C) or high (over 60°C) temperature shift isolation.[1] Thus, no problem appears to exist in the isolation of saprophytic *Bacillus* species from nature.

However, there is a problem in the isolation of minor populations of *Bacillus* strains from a soil sample in which many other spores of *Bacillus* species predominate. *Bacillus cereus, B. cereus* var. *mycoides, B. sphaericus,* and *B. subtilis* often occur as major populations in soil samples[3~9] while many other *Bacillus* species such as *B. circulans, B. brevis* and *B. laterosporus* usually occur as minor populations. Using standard dilution-plate technique, it is difficult to isolate such minor *Bacillus* strains on agar plates. In antibiotic production, there is no superiority or inferiority between the major and minor populations. Therefore, the selective isolation of such minor *Bacillus* strains would be useful to increase the efficacy of antibiotic screening.

This work was initiated to obtain minor *Bacillus* efficiently from soil samples. Spores of most predominant *Bacillus* strains such as *B. cereus* germinate and grow during a short period of incubation on a

germination medium which usually contains L-alanine[10] and mono- or di-valent cations,[11] but spores of minor *Bacillus* strains such as *B. circulans* remain dormant under these conditions in general. The minor group of bacteria is enriched by heating the incubated mixture at 65°C for 10 minutes. The isolation procedure described here is based on this principle and named "minor-shifted isolation". WOOD and CASIDA[12] have reported an enrichment technique for isolating group II *Bacillus* species from soil samples. They enriched the *Bacillus* strains by incubating the soil sample in a glucose-minerals solution for 6 days, followed by heating it at 100°C for 30 minutes.

Materials and Methods

Bacillus Strains Used

Thirty-six strains of *Bacillus* (see Table 1) were used in this study. They were obtained from Institute for Fermentation, Osaka (IFO), University of Osaka, School of Technology (OU), American Type Culture Collections (ATCC), Northern Regional Research Laboratory (NRRL), and collections of our laboratory.

Measurement of Germination Rate

To prepare spore suspensions for the germination test, each *Bacillus* strains was cultivated on glycerol-isolation agar medium (Gly-IM agar)* for 4 to 7 days at 30 to 35°C. The growth paste was suspended in sterile saline and treated at 65°C for 10 minutes. After cooling, the suspension was diluted to about 10^5 colony forming units (CFU) per ml.

The germination medium contained 1.0% glucose, 0.5% Casamino Acids, 0.3% beef extract, 0.1% yeast extract, and 0.1% DL-alanine (pH 6.8, 110°C for 15 minutes). Two-tenth ml of the spore suspension and 4.8 ml of the germination medium were combined and incubated in a water bath at 30 or 35°C for 0.5 to 6 hours under gentle shaking. At various time intervals, 0.2 ml of the mixture was taken out and combined with 1.8 ml of sterile saline, and then immediately treated at 65°C for 10 minutes to kill the germinated *Bacillus* cells. After cooling, 0.2 ml of the mixture was taken out and used to count the surviving spores by the standard dilution-plate method. The germination ratio at each time interval was calculated as follows: [(Dormant spores at 0 hour)−(Dormant spores at an incubation interval)] ÷ (Dormant spores at 0 hour)×100%.

"Minor-shifted Isolation" Technique

A dried soil sample (about 0.5 g) was suspended in 5 ml of sterile saline and stirred with three to five glass beads (about 4 mm in diameter) by a Micro Thermo-Mixer (Model TM-101, Thermonics Co., Ltd., Tokyo) for 1 minute. Next, the suspension was placed in a vacuum desicator for about 30 minutes in order to eliminate air from the mixture. The vacuum treated sample was then heated at 65°C for 10 minutes to prompt the germination of *Bacillus* spores in the sample and to diminish various kinds of bacteria other than *Bacillus*.

One milliliter of the above soil suspension and 1.0 ml of sterile germination medium were combined aseptically and incubated at 30°C for 2 to 3 hours with gentle shaking (90 rpm). The incubated mixture was then treated at 65°C for 10 minutes to kill the germinated *Bacillus*. Upon cooling, the mixture was diluted serially and 0.5 ml was plated on Gly-IM agar plates. Generally, 1-, 10-, and 100-fold dilutions of the mixture were used. Colonies were isolated from the plates after incubation at 30°C for 2 to 12 days.

Results

Spore Germination of Various *Bacillus* Species

Thirty six strains of *Bacillus* species were tested for their germination rate as described in "Materials

* Gly-IM agar: 0.2% w/v soluble starch, 0.2% glycerol, 0.25% polypeptone, 0.25% yeast extract, 0.25% beef extract, 0.3% NaCl and 1.25% agar, pH 6.8.

Table 1. Germination ratios of various *Bacillus* spores[a].

No.	Strains	°C	Germination ratio (%)					
			0	0.5	1.0	2.0	3.0	6.0 hours
1	*B. cereus* IFO 3131	30	0	100	100	100	100	100
2	*B. cereus* IFO 3001	30	0	100	100		100	100
		35	0	100			100	100
3	*B. cereus* IFO 3466	30	0				100	100
4	*B. cereus* IFO 3342	30	0	100	100		100	100
5	*B. cereus* JP-25	30	0	100	100		100	100[c]
6	*B. cereus* CB-35	30	0				100	100
7	*B. cereus* Gp-3	30	0	75.6	75.6			31.7
8	*B. cereus* 60–6	30	0	83.8	89.9	99.5	99.6	98.5
9	*B. megaterium* IFO 1035	30	0	99.7	100		100	100
		35	0		97.9	99.2	99.6	99.8[c]
10	*B. megaterium* 67	30	0	90.0	97.0	97.9		99.0[c]
11	*B. megaterium* I-13 (19)	30	0	4.8	0	0	40.5[b]	66.7
12	*B. subtilis* IFO 36061	30	0	95.0	90.0	90.0	100[b]	100
13	*B. subtilis* 78	35	0		60.0	84.0	80.0	82.0[c]
14	*B. subtilis* AR-30	35	0		6.3	50.0	62.5	66.7[c]
15	*B. subtilis* IFO 3007	30	0	97.0	98.0	97.0	99.5	99.5
16	*B. pumilus* IFO 3813	35	0		22.7	45.5	54.5	77.3[c]
17	*B. pumilus* 61	35	0		76.0		96.0	97.0
18	*B. pumilus* TL-47	30	0	24.4	24.4	21.7	50.0[b]	47.8
		35	0	74.4	82.1	82.1	92.3[b]	94.1
19	*B. pumilus* 37	30	0	17.1	8.6	17.1	35.7[b]	38.6
		35	0		19.2	30.8	46.2	63.8[c]
20	*B. licheniformis* OU 8356	35	0		86.1	86.1	95.0	96.1[c]
21	*B. circulans* NRRL B3313 (14)	30	0		0		0	0
		35	0		0		16.1	16.1
22	*B. circulans* OU 8356	30	0		68.5	67.7	77.7	70.0
		35	0		79.2		79.2	75.0
23	*B. circulans* 57–33	30	0	24.5	46.1	50.6	91.0	92.7
		35	0		30.0	60.0	68.0	71.0
24	*B. brevis* Ak-4	30	0	18.8	25.0	18.8	31.3	37.5
25	*B. brevis* OU 3864	30	0		95.3	98.5	98.6	98.9
		35	0		73.8		87.7	84.6
26	*B. laterosporus* 340–19	30	0		56.4	72.7	72.7	86.4[c]
		35	0		86.2		96.0	98.0[d]
27	*B. pulvifaciens* CB-57	30	0		5.6	5.6	11.1	55.6
		35	0		0		0	50.0
28	*B. polymyxa* AR 110	35	0		66.9	53.8	50.0	89.2
29	*B. polymyxa* RS-6	35	0		0	0	0	62.0
30	*B. coagulans* OU 3843	30	0		0	0	0	58.3
		35	0		0		50.0	88.0
31	*B. coagulans* OU 8344	30	0		33.3	60.0	50.0	43.3
32	*B. sphaericus* ATCC 7055	30	0		92.8	95.0	97.6	98.3
		35	0		50.0		56.5	69.6

Table 1. (Continued)

No.	Strains	°C	Germination ratio (%)					
			0	0.5	1.0	2.0	3.0	6.0 hours
33	*Bacillus* sp. No. 45 (rhizoidal)	30	0	100	100	100	100[b]	100[d]
34	*Bacillus* sp. No. 42 (spread)	30	0		100	100	100[b]	100
35	*Bacillus* sp. No. A (rhizoidal)	35	0		98.0	98.0	97.0[b]	98.2[d]
36	*Bacillus* sp. No. B (spread)	35	0		0		96.0	97.0

[a] Assay conditions: Each 10^2 to 10^5 per ml of spore suspension was incubated at 30 or 35°C for 0.5 to 6 hours under gentle shaking. The germination ratio at each interval was calculated as described in Materials and Methods. Germination ratio=[(Spores at 0 hour)−(Spores at an incubation interval)] ÷(Spores at 0 hour)×100%; [b],[c] and [d]: Data at 4, 5 and 7 hours stage, respectively.

and Methods". The results obtained are shown in Table 1.

B. cereus strains tested were commonly rapid and uniform germinators. Among the eight strains tested, five of *B. cereus* strains germinated 100% of their spores within 0.5 hour of incubation. Other three strains of *B. cereus* also germinated over 75% of their spores within 0.5 hour. Cerexin B-producing *B. cereus* Gp-3[18] was an exceptionally slow and/or uneven germinator with about 20% of the spores remaining in dormant form after 2 to 3 hours of incubation.

B. megaterium and *B. subtilis* strains tested had divided results. Only two of three *B. megaterium* strains and two of four *B. subtilis* strains germinated over 90% of their spores within 0.5 hour of incubation.

B. pumilus, B. circulans, B. brevis, B. laterosporus, B. pulvifaciens, B. polymyxa and *B. coagulans* strains were generally slow and/or uneven germinators with over 15% of their spores remaining dormant after 2 to 3 hours of incubation. The only exception was *B. brevis* OU 3804 which was a rapid and uniform germinator.

B. sphaericus strains, *Bacillus* sp. 42 and 61 (probably *B. sphaericus* which occur frequently in soils and makes isolation of other *Bacillus* colonies difficult by their spreading growth over the plates) were all rapid and uniform germinators. *Bacillus* sp. 45 and 78 (probably *B. cereus* var. *mycoides* which frequently disturb the colony formation of other *Bacillus* strains by their rhizoidal spreading growth over the isolation plates) were also all rapid and uniform germinators.

These results indicate that most of the major populations of *Bacillus* are rapid and uniform germinators and most of the minor populations of *Bacillus* species are slow and/or uneven germinators. Therefore, the minor populations of *Bacillus* can be enriched by incubating the dried soil samples in the appropriate germination medium for 2 to 3 hours followed by treatment at 65°C for 10 minutes.

To determine the effect of germination temperature, several rapid and slow germinators of *Bacillus* were incubated at both 30 and 35°C (see Table 1). *B. pumilus* TL-47 germination was somewhat efficient at 35°C but *B. sphaericus* ATCC 7055 was not. No significant difference was observed on the other strains (see Table 1). Therefore, it is considered that both 30 and 35°C incubations are useful for "minor-shifted isolation". We used 30°C germination in this study.

Two Model Experiments of "Minor-shifted Isolation"

As model experiments, the following two spore mixtures were used for the "minor-shifted isolation".

Table 2. Two model experiments of "minor-shifted isolation".

Mixture	Strains	No. spores (CFU/0.2 ml)					
		Standard dilution-plate method			Minor-shifted isolation method		
		0	0.5	3 hours	0	0.5	3 hours
I	*B. cereus* IFO 3466	2400	11400	12800	2400	26	8
	B. circulans NRRL B3313	3	0[a]	0[a]	3	6	5
II	*B. cereus* IFO 3001	6100	4000	12800	6100	175	50
	B. polymyxa AR-110	24	0[a]	0[a]	24	40	19

[a] No colony could be detected.

Mixture I contained in 0.2 ml, 2.4×10^3 *B. cereus* IFO 3466 spores and 3 *B. circulans* NRRL B3313[14] spores. Mixture II had 6.1×10^3 *B. cereus* IFO 3001 spores per 0.2 ml and 24 *B. polymyxa* AR-110[15] spores per 0.2 ml. The isolations were done as described in "Materials and Methods" and the results are shown in Table 2. For comparison, the standard dilution-plate method was also used for these mixtures (see Table 2).

In both experimental mixtures, colony formation of the minor components, *i.e. B. circulans* NRRL B3313[14] and *B. polymyxa* AR-110[15] could not be detected by the standard dilution-plate method due to interference by the major component colonies. However, "minor-shifted isolation" allowed formation of clear detectable colonies of the minor *Bacillus* component (see Table 2).

The results, therefore, indicate that the "minor-shifted isolation" procedure makes possible enrichment for minor, slow and/or uneven germinating *Bacillus* strains from among 250- to 800-fold more of the major, rapid germinators.

"Minor-shifted Isolation" from Four Soil Samples

"Minor-shifted isolation" from four soil samples was compared with the standard dilution-plate method. The soil samples were obtained from the Okinawa-Iriomote district, Japan. The isolation procedure is described in Materials and Methods. The taxonomic examination of the isolated strains was done in accordance with BERGEY's Manual of Determinative Bacteriology, 8th Edition. The same species strains obtained from a soil sample by an isolation method were compared their appearances on agar slants, and identically appearing strains were combined as one strain. The results obtained are listed in Table 3.

B. cereus, B. cereus var. *mycoides* and *B. megaterium* strains were obtained from all A, B, C, and D soil samples by the standard method. Colonies of these species strains usually covered the isolation plates as predominant populations. They were however clearly diminished by the "minor-shifted isolation". No comparisons have been done between the standard group and the "minor-shifted" group of isolates in the same species. This means the same strains may be divided into both groups of isolates. *B. pumilus* and *B. sphaericus* strains also showed similar tendency as the above species. In contrast, *B. circulans* and *B. brevis* strains were isolated more frequently by the "minor-shifted" method. The *B. circulans* strains were obtained from all the A, B, C, and D soil samples by the "shifted" method but were not isolated from the A, B, and D soil samples by the standard method. The result indicates that the "minor-shifted" method is definitely efficient for isolation of *B. circulans* strains. *B. brevis* strains showed the same behaviour to *B. circulans*. *B. subtilis, B. polymyxa, Bacillus* sp. (*megaterium*-like),

Table 3. Comparison between "minor-shifted isolation" and standard dilution-plate method with four soil samples.

No.	Species	Soil No.[a]									
		Standard isolation					Minor-shifted isolation				
		A	B	C	D	Total	A	B	C	D	Total
1	*B. cereus*	1	11	3	1	16	1	1	1	0	3
2	*B. cereus* var. *mycoides*	2	1	1	1	5	0	1	0	0	1
3	*B. megaterium*	5	4	3	1	13	2	0	0	1	3
4	*B. subtilis*	0	0	0	0	0	0	0	1	0	1
5	*B. pumilus*	1	5	4	1	11	1	2	4	0	7
6	*B. licheniformis*	1	0	0	0	1	4	0	0	0	4
7	*B. firmus* or *lentus*	5	0	0	0	5	2	0	0	0	2
8	*B. polymyxa*	3	0	0	0	3	0	0	2	1	3
9	*B. brevis*	3	0	2	0	5	1	2	6	1	10
10	*B. circulans*	0	0	1	0	1	2	2	3	5	12
11	*B. pulvifaciens*	0	0	1	0	1	0	0	0	0	0
12	*B. sphaericus*	1	5	0	1	7	0	1	0	4	5
13	*Bacillus* sp. (*cereus*-like)	0	0	1	1	2	1	0	1	0	2
14	*Bacillus* sp. (*megaterium*-like)	0	0	0	2	2	1	2	0	2	5
15	*Bacillus* sp. (*circulans*-like)	0	0	0	0	0	0	0	0	1	1
16	*Bacillus* sp. (*firmus-lentus*-like)	0	0	0	0	0	0	0	1	0	1
17	*Bacillus* sp. (unknown)	1	0	0	0	1	0	0	0	1	1

[a] The four soil samples used in this isolation were signified as A, B, C and D.

Bacillus sp. (*circulans*-like), and *Bacillus* sp. (*firmus-lentus*-like) strains were also isolated by the "minor-shifted" method from soil samples from which none of these strains were obtained by the standard method. Other species strains were isolated with similar or uncertain frequencies by the both methods.

The above results indicate that the standard dilution-plate method is suitable for isolation of *B. cereus*, *B. cereus* var. *mycoides*, *B. megaterium*, *B. pumilus* and *B. sphaericus* (predominant populations) and the "minor-shifted" method is efficient for obtaining *B. circulans*, *B. brevis*, *B. subtilis*, *B. polymyxa*, and three unidentified groups of strains (see Table 3) (minor populations). This also means the "minor-shifted" method is a practical way to enrich the minor *Bacillus* populations in soil samples.

Discussion

Many kinds of selective isolation procedures have been reported for bacteria, especially for bacteria of clinical importance.[16~23] The antibiotic agar method is generally used for selective isolation.[19,20] However, it is not applicable to *Bacillus* strains since no antibiotics have been reported to be selective for *Bacillus* strains over other Gram-positive bacteria. Moreover, antibiotic agar isolation is an uncertain method for selecting special antibiotic producers. For example, gentamicin-producing *Micromonospora* strains are not always resistant to gentamicins, and edeine-producing *Bacillus* strains are resistant not only to edeine but also to kanamycin and neomycin.

Electrophoretic[17] and ion-exchange resin[23] separation of *Bacillus* spores would be difficult because of their strong adsorption to soil particles.[24,25]

The "minor-shifted isolation" method makes it possible to obtain minor, slow and/or uneven germinating *Bacillus* strains from soil samples which contain about 1,000-fold more spores of rapid and uni-

form germinators of *Bacillus*. To protect leaking of glucose-retardatory *Bacillus* such as *B. firmus* and *B. lentus*, Gly-IM medium is used in this isolation. Some modifications are necessary to isolate certain species of *Bacillus* by this method. For example, addition of 2-phenylacetamide and fructose to germination medium is necessary for *B. macerans* strains,[2] and high (55~60°C) or low (10°C) temperature shift is essential for thermophilic or psychrophilic *Bacillus* species.

This isolation technique has another merit in the isolation of *Bacillus*. The standard dilution-plate technique is frequently accompanied by trouble from spreading and/or rhizoidal growth of *Bacillus* strains over the agar plates. Since most of the spreading strains are rapid and uniform germinators, this "minor-shifted" method usually eliminates such troublesome strains and results in the formation of well-separated colonies of the minor *Bacillus* strains.

A weak point of this method is that it cannot eliminate some strains of *B. pumilus, B. sphaericus, B. megaterium* and *B. subtilis* which occur as predominant populations. Some of these *Bacillus* strains are rapid and uniform germinators but the remainders are not. Elimination of the latter group is, therefore, not achieved by this method.

Consideration of the merits and demerits of this technique showed that it is still useful for isolating certain *Bacillus* strains from natural sources. In parallel with the standard dilution-plate method, this technique was valuable for antibiotic screening.

We have obtained the following 19 new antibiotics from *Bacillus* strains by use of this "minor-shifted" method with the standard method: cerexin A and B from *B. cereus* 60-6 and Gp-3, respectively,[13] galantin A and B from *B. pulvifaciens* 52–33,[26] antibiotic TL-119 from *B. subtilis* TL-119,[27] antibiotic 61–26 from *Bacillus* sp. 61–26,[28] thiocillin I, II and III from *B. badius* AR-91, *B. cereus* G-15 and *B. megaterium* I-13, respectively,[29] antibiotic 339–29 from *B. pumilus* 339–29,[30] antibiotic 333–25 from *B. circulans* 333–25,[31] brevistin from *B. brevis* 342–14,[32] antibiotic B-43 from *B. circulans* B-43,[33] polymyxin S_1 and T_1 from *B. polymyxa* RS-6 and E-IE, respectively,[34] and tridecaptin A, B and C from *B. polymyxa* AR-110, B-2 and E-23, respectively.[15]

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