Korean J. Appl. Microbiol. Bieong
Vol. 4, No. 4, 166~178 (1976)

DNA-DNA Hybridization에 의한 *Bacillus coagulans*의 분류학적 연구

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Taxonomic Study of *Bacillus coagulans* by Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization Technique

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(Received October 11, 1976)

Abstract

Taxonomic study of 11 strains of *Bacillus coagulans* and 14 strains of 13 species of *Bacillus* by deoxyribonucleic acid (DNA)-DNA hybridization were conducted. Among the 11 strains of *B. coagulans*, 6 were isolated from soil and the rest were the authentic strains obtained from American Type culture collection (ATCC) or the Institute for Fermentation, Osaka (IFO). All strains were examined to confirm as they are expected species of *B. coagulans* by the methods of Gordon *et al.* according to Bergey's Manual (8 th ed.).

The intraspecific DNA homology indexes among the 11 strains of *B. coagulans* using strain ATCC 7050 as the standard (^3H labeled input DNA) showed 76 % or, more, respectively. These findings accorded well with the results of the conventional taxonomic study according to the Bergey's Manual.

The interspecific DNA homology indexes between *B. coagulans* strain ATCC 7050 and the type cultures of *B. subtilis* (168), *B. licheniformis* (IFO 12107), *B. pumilus* (IFO 12110), *B. firmus* (ATCC 14575), *B. lentus* (ATCC 10840), *B. circulans* (ATCC 4513), *B. macelans* (ATCC 8244), *B. polymyxa* (ATCC 842), *B. sphaericus* (ATCC 14577), *B. brevis* (ATCC 8246, IFO 12334), *B. laterosporus* (ATCC 64), and *B. pantothenticus* (ATCC 14576) respectively, showed 2 to 4%, while that of between *B. coagulans* ATCC 7050 and *Escherichia coli* K-12 was less than 1 %.

Introduction

The Genus *Bacillus* was divided into three groups by R. E. Gordon *et al.* [1] (Fig. 1). Group I strains including *B. megaterium*, *B. cereus*, *B. subtilis*, *B. licheniformis*. *B. pumilus*, and *B. firmus* were distinguished from other two groups because their sporangia do not definitely swell. Group 3 including *B. sphaericus* was separated from Group 2 because of spherical shape of its spores. *Bacillus coagulans* was located on the line dividing the species of Group I and Group 2 because these strains have both type of sporangia; not defenitely swollen sporangia and swollen one. Therefore, hereby it was required to investigate genetic relat-

Table 1. List of Strains used

Strains	Remarks
Bacillus coagulans	
ATCC[a] 7050	←N. R. Smith, 609
IFO[b] 3557	←T. Harada (Institute of Scientific and Industrial Research)
IFO 3886	←RIFY[c] (O. Nakayama, P22)
IFO 3887	←RIFY (O. Nakayama, A22)
IFO 12583	←IAM[d], 1115
C-1	Isolated from soil
C-2	Isolated from soil
C-3	Isolated from soil
C-4	Isolated from soil
C-5	Isolated from soil
C-6	Isolated from soil
B. subtilis 168	←S. Yuki←J. L. Farmer and F. Rothman
B. licheniformic IFO 12107	←ATCC 9945a←C. B. Thorne
B. pumilus IFO 12110	←NRRL[e] B-1489←N. R. Smith, 236
B. firmus ATCC 14575	←R. E. Gordon←N. R. Smith, 613←Wender
B. lentus ATCC 10840	←N. R. Smith, 670←T. Gibson, 165
B. circulans ATCC 4513	←W. W. Ford, 26
B. macelans ATCC 8244	←N. R. Smith, 888←F. Schardinger
B. polymyxa ATCC 842	←A. J. Kluyver
B. sphaericus ATCC 14577	←R. E. Gordon←T. Gibson, 1030←E. Neide
B. brevis ATCC 8246	←N. R. Smith, 604←W. W. Ford. 27B
B. brevis IFO 12334	←ATCC 10027←N. R. Smith←G. Bredemann
B. laterosporus ATCC 64	←AMC[f] 797←W. W. Ford, 29←Laubachi
B. pantothenticus ATCC 14576	←R. E. Gordon←N. R. Smith, 1321←H. Proom
Escherichia coli K-12	Stock culture in Osaka Univ.

a American Type Culture collection, Rockville, Md
b Institute for Fermentation, Osaka, Japan
c Research Institute of Fermentaion, Yamanashi Univ., Kofu, Japan
d Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
e Northan Utilization Research and Development Division, U. S. Dept. of Agriculture, Peoria, Ill.
f Walter Read Army Medical Center, Washington, D. C.

ionships of *B. coagulans* at molecular level. Although many comparison of genetic exchange of *Bacillus* species have been made until now, no informations on the genetic homologies among related DNAs of *B. coagulans* by DNA-DNA hybridization method have been made for a quantitative measure of homologies with the membrane-filter technique [9].

In present paper taxonomic study by DNA-DNA hybridization method was applied to differentiate DNA homologies among the various strains of *B. coagulans* and between *B. coagulans* and other species of *Bacillus*. And the results obtained by DNA-DNA hybridization were also compared with these of the conventional taxonomic study.

Materials and Methods

Strains The strains used in this study are listed in Table 1. Among the 11 strains of *B. coagulans*, 5 were obtained from authentic sources and 6 strains were isolated from soil in Osaka Prefecture, Japan. The taxonomic significance of all strains except *B. coagulans* IFO 12583 were examined according to the method described in the Genus *Bacillus* by R. E. Gordon *et al* [1]. All strains

were stored at 5 °C on PGY agar slants (polypeptone [Daigo Eiyo]; 1% (w/v), glucose; 1 %, yeast extract [Daigo Eiyo]; 1 %, agar; 1.5 %, pH 7.0), supplemented with 200 μg of thymine per m*l* if necessary.

Streptomycin resistant mutant was isolated spontaneously from *B. coagulans* strain ATCC 7050. The resistant mutant was capable of growing on PGY agar medium containing 600 μg of streptomycin per m*l*. Thymine auxotrophic mutants from streptomycin resistant mutant of strain ATCC 7050 (Str^R) were prepared by cultivating cell in medium which 50μg of 2, 4-diamino-5-(3'4'4'-trimethoxy)-benzylpyrimidine (trimethoprim; Sigma) and 200 μg of thymine per ml respectively were added to modified Spizizen minimum medium[7] (MSM medium; Table 2) according to Stacey and Simson[8]

Table 2. Modified Spizizen Minimum Medium (MSM Medium) used in this Study.

Glucose	5g/*l*
$MgSO_4$	200mg/*l*
Casamino acid	5g/*l*
L-Tryptophane	50mg/*l*
Vitamines;	
d-Biotin	1mg/*l*
Thiamine	1mg/*l*
Nicotinic acid	1mg/*l*
Spizizen minimum salt solution;	
K_2HPO_4	14g/*l*
KH_2PO_4	6g/*l*
$(NH_4)_2\ SO_4$	2g/*l*
Na-citrate	1g/*l*

Since almost all colonies isolated as thymine auxotrophs required more than 25μg of thymine per m*l* for growth, appropriately diluted cell suspensions were plated on MSM medium containing 2 μg of thymine per ml. Colonies appeared spontaneously on the plate were picked up and it was confirmed that they were able to grow on MSM medium containing 2 μg of thymine per m*l*. All medium used to examine morphological and physiological features, depended upon Genus *Bacillus* by R. E. Gordon *et al.*[1] if otherwise describe. During preparation of MSM medium Spizizen minimum solution was used with a 10-fold dilution.

Media and Growth Conditions for Microorganisms of DNA Preparation For cultivation of *B. coagulans*, cells grown overnight at 48 °C in 5 m*l* PGY broth were inoculated into 100 m*l* of fresh PGY medium and incubated with shaking culture for 10 hrs. 70 m*l* of precultured broth were transferred and incubated at 48 °C in 1 liter of fresh PGY medium with aeration in an orbital incubater (100 rpm). As strains C-2 and C-4 showed poor growth in PGY medium, meat extract (0.2 %) was added to the PGY medium. Cells at late logarithmic phase in the culture were harvested by centrifugation at 7,000 rpm for 10 minutes (depending upon strains, medium and inoculum size), and were used for DNA extraction. Radioactive DNA was prepared from the cells of thymine requiring mutant of *B. coagulans* ATCC 7050 (Str^R-thy) and *B. subtilis* 168 (Str^R, thy) cultivated in MSM medium containing 2μg of [methyl-^{3}H]-thymine (1.25μCi/g; the Radio Chemical Center Ltd., Amersham, England).

DNA Extraction and Purification DNAs from all strains were prepared by the method of Marmur[2] with partial modification[4] as described by Seki *et al.* For elimination of RNA from the crude DNA preparation, pancreatic ribonuclease (Sigma), of which the deoxyribonuclease had been inactivated by heating at 80 °C for 10 minutes, was used. The DNA obtained was kept in a solution of 1× SSC (SSC; 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) at 5 °C. The specific radioactivity of purified tritium labeled DNA was about 40,000 cpm per μg. DNAs from the species other than *B. coagulans* and ^{3}H labeled DNA from *B. subtilis* 168 were given by Mr. Kawai (Dept. of Fermentation Technology, Faculty of Engineering, Osaka University, Osaka, Japan).

Quantitative Determination of DNA The amount of DNA was measured by the method of Burton[10], using 2-deoxyadenosine monophosphate as standard. To 20 m*l* of the diphenylamine reagent (1.5 g of diphenylamine is dissolved in a 100 m*l* acetic acid solution containing 1.5 m*l* of concentrated H_2SO_4) 0.1m*l* of 1.6 % acetoaldehyde was added. One m*l* of adequately diluted DNA solution

was added to 2 ml of the above reagents and the mixture was allowed to stand for 18 hrs. at 30°C. The optical density was measured at 600 nm. The radioactivity of DNA solution was measured according to the method of Seki *et al* [4].

Preparation of DNA filter and DNA-DNA Hybridization The DNA filter was prepared according to the method of Gillespie and Spiegelman [9] with partial modification [4]. The amount of DNA immobilized on a membrane filter (disk DN-A) was adjusted to 25 μg. The procedure for DNA-DNA hybridization followed the method described by McConaughy *et al.* [11] with partial modification [4]. Disk DNAs were incubated with 0.5 μg of tritium labeled solution (input DNA) in 1 ml solution of 6×SSC-*formamide* (50 %) for 22.5 hrs at 37° C. After DNA-DNA hybridized disk filters were washed with 3mM tris hydroxymethyl aminomethane hydrochloride buffer (pH 9.0) and dried in a vacuum oven for about 1 hour, the amount of input DNA bound to the disk DNA was estimated by counting the radioactivity of the filter for 1 minute using a liquid scintillation counter (Beckman model LS-250).

Results

1. Identification of isolated strains Table 3 summarized the morphological and physiological features of 10 strains of *B. coagulans*.

In order to isolate *B. coagulans* from soil, 1 gram of soil was sampled and heated in a 80 °C water bath to remove vegitative cell because it was known that *B. coagulans* existed with spore-forming in soil. As the optimum temperature of growth of *B. coagulans* is above 50 °C, strains capable of growing on nutrient agar plate at 50 °C were first selected. Physiological properties of these selected strains were next examined in nutrient broth medium containing 0, 2, 5, or 7 percent (w/v) of NaCl. Some strains capable of growing in 2 % NaCl broth, but not in 5 % NaCl broth were selected at the temperature of 48 °C after 2-5days' incubation. Those selected strains were again tested the ability of coagulation of milk medium (city milk was used after 10 minute centrifugation at 3,000 rev/min to reduce the fat content). The selected strains here were once more checked in litmus milk (method by R.E. Gordon *et al.*) to observe acid reaction of litmus, reduction of litmus and curd. In this experiment strain C-2 showed no curd in litmus milk, though showing curd in city milk.

On microscopic appearance, cells grown on PGY slants for 15 to 24 hours or longer if required were used for the observation of shape, size, motility, gram reaction. sporangia or no sponrangia of their spores. All tested strains showed ellipsoidal shape

Table 3. Taxonomical Characteristics of the Test Strains of *B. coagulans*

Strains	Optimum temp. (°C)	Growth in NaCl (5%) broth	Growth in NaCl (2%) broth	Litmus milk
ATCC 7050	50	−[a]	+[b]	ARC[c]
IFO 3886	50	−	+	ACR
IFO 3887	50	−	+	ACR
IFO 3557	50	−	+	ACR
C-1	50	−	+	ACR
C-2	50	−	+	AR
C-3	50	−	+	ACR
C-4	55	−	+	ACR
C-5	50	−	+	ACR
C-6	50	−	−	ACR

a Growth was negative.
b Growth was positive.
c A; Acid reaction of litmus, C; Curd, R; Reduction of litmus

Table 3. continued

Strains	Size of cell	Size of spore	Shape of spore	Sporangia of spore	Motility	Gram reaction
ATCC 7050	1.0[a] 3.75−5.0[b]	1.0−1.25 1.25−1.5	ellipsoidal	swollen	+	+
IFO 3886	0.75−1.0 5.0−7.5	1.0 1.25−1.5	ellipsoidal	swollen	+	+
IFO 3887	0.75−1.0 3.75−10.0	1.0 1.25−1.5	ellipsoidal	swollen	+	+
IFO 3557	0.75−1.25 5.0−6.25	1.0−1.25 1.25−1.5	ellipsoidal	swollen	+	+
C-1	0.75−1.0 3.75−5.0	1.0−1.25 1.25−1.5	ellipsoidal	swollen	+	+
C-2	0.75−1.0 3.75−4.0	1.0 1.25−1.5	ellipsoidal	swollen	+	+
C-3	0.75−1.0 3.75−6.25	1.0−1.25 1.25	ellipsoidal	swollen	+	+
C-4	0.75−1.0 3.75−5.0	1.0−1.25 1.25	ellipsoidal	swollen	+	+
C-5	1.0 3.75−5.5	1.0−1.25 1.5	ellipsoidal	swollen	+	+
C-6	1.0 3.75−6.25	0.75−1.0 1.5−1.75	ellipsoidal	swollen	+	+

a Upper line is diameter.
b Lower line is length.

(**Table 3.** continued)

Strains	V-P reaction	pH in V-P broth	Catalase	Anaerobic growth	Egg-yolk reaction	Lyzozyme resistance	Growth in pH 5.7 medium
ATCC 7050	+	4.4	+	+	−	−	+
IFO 3886	+	5.3	+	+	−	−	+
IFO 3887	+	5.3	+	+	−	−	+
IFO 3557	+	5.1	+	−	−	−	+
C-1	+	5.5	+	+	−	−	+
C-2	+	4.8	+	±	−	−	+
C-3	+	4.8	+	+	−	−	+
C-4	+	4.7	+	+	−	−	+
C-6	+	N.G	+	+	−	−	+

(**Table 3.** continued)

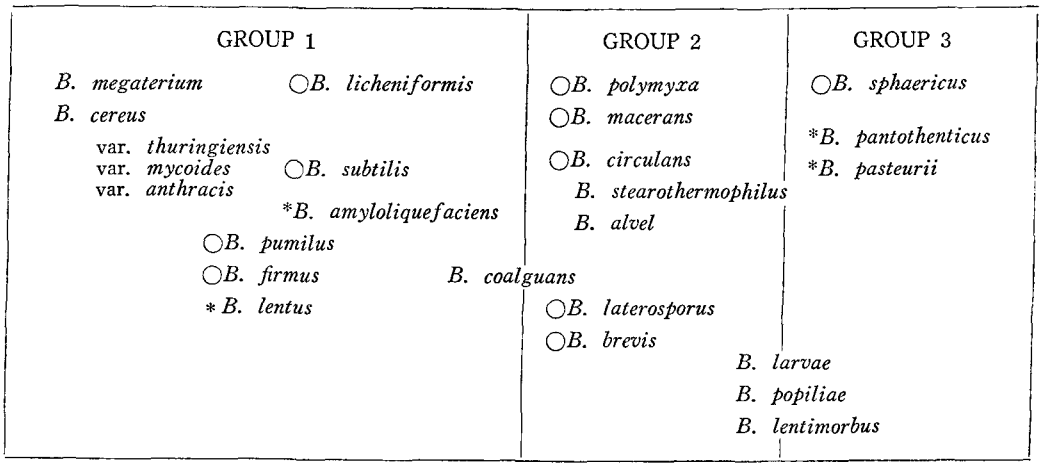
Strains	Growth in azide (0.02%)	Acid from glucose	arabinose	xylose	mannitol	Hydrolysis of starch	NO_2 from NO_3
ATCC 7050	+	+	−	+	−	+	N.G
IFO 3886	N.G[a]	+	−	+	+	+	N.G
IFO 3887	N.G	+	−	+	−	+	N.G
IFO 3557	−	N.G	−	N.G	−	+	N.G
C-1	N.G	+	−	+	+	+	N.G
C-2	N.G	+	−	+	−	+	−
C-3	N.G	+	−	+	+	+	N.G
C-4	N.G	+	−	+	+	+	N.G

C-5	N. G	N. G	−	N. G	−	+	N. G
C-6	N. G	+	−	+	−	+	N. G

a In the test of growth in azide, the growth of cell did not observed in the medium without azide, and in other test, N. G. showed no growth in the medium used.

(Table 3. continued)

Strains	Use of citrate	Use of propionate	Formation of dihydroxy-acetone	Formation of indole	Deamination of phenylalanine	Decomposition of casein	Decomposition of tyrosine
ATCC 7050	−	−	N. G	−	−	−	−
IFO 3886	−	−	N. G	−	−	−	−
IFO 3887	−	−	N. G	−	N. G	−	−
IFO 3557	−	−	N. G	−	N. G	−	−
C-1	−	−	N. G	−	−	−	−
C-2	−	−	N. G	−	N. G	−	−
C-3	−	−	±	N. G	N. G	−	−
C-4	−	−	N. G	N. G	−	−	−
C-5	−	−	N. G	N. G	−	−	−
C-6	−	−	±	−	−	−	−



Strains marked with used in this study and strains marked with *are not enrolled in Bergey's manual 8th edition.

Fig. 1. Genus *Bacillus* divided into 3 Groups by R. E. Gordon et al. (1973).

and swollen sporangia of spore. Therefore it was thought that these strains might, morphologically, belong to Group 2 (sponrangia swollen by oval spores) according to Genus *Bacillus* divided into 3 groups by Gordon *et al.* (Fig. 1). During test of anaerobic growth, strains IFO 3557 showed no growth along the length of the stab, but growth on the surface of the agar when a loopful of culture broth precultured in nutrient broth for 16 hours was stabbed to the bottom of a tube of anaerobic agar (medium composition by R. E. Gordon *et al.*). Accordingly although one percent of yeast extract was supplemented to Gordon's anaerobic medium, no growth showed along the stab, either. And strain C-2 presented scanty growth to the anaerobic medium. For the test of resistance at 0. 02 percent sodium azide, all strains tested showed no growth except for strain ATCC 7050 as control.

Therefore although cells grown in PGY broth medium inoculated once more in PGY medium containing sodium azide 0.2 gram per liter, they showed no growth, either. Accordingly althouth strains tested showed no growth during physiological experiments such as resistance to sodium azide, reduction of nitrate to nitrite and production of dihydroxyacetone, in present paper it was not deeperly investigated the nutritional requirement properties concerning reaction of "no growth", owing to being not key points to *B. coagulans*. With these results of morphological and physiological test according to the conventional taxonomic study it was indicated as follows: the newly isolated 6 strains were identified as *B. coagulans*, but actually these 10 strains of *B. coagulans* including authentic type cultures were morphologically much nearer to Group 2 because they have swollen sporangia and ellipsoidal shape of spores. Therefore, hereby it was required to investigate genetic relationships among these strains at molecular level.

2. Improvement of strain for preparing the labeled DNA

(A) Isolation of streptomycin-resistant strains

Isolation of resistant strains streptomycin to eliminate contamination with other bacteria were required to get pure labeled DNA from *B. coagulans* strain ATCC 7050. Cells grown in PGY liquid medium at 48°C for 18 hours were plated on PGY agar medium containing 0.5 mg or one mg of streptomycin per m*l*, but no growth on plates was observed. Accordingly some colonies capable of growing spontaneously at low concentration of 10 μg of streptomycin per m*l* were first isolated from parent strain. By replicating these colonies on PGY medium containing the various stepped-up concentration of streptomycin. Some colonies capable of growing at the concentration of one mg of streptomycin per m*l* were isolated, but they showed scanty growth. For preparing labeled DNA, actually colonies capable of growing at the concentration of 600 μg of streptomycin per m*l* were used. Colonies isolated here were checked their coagulations in milk medium to observe whether these were contaminated or not.

(B) Isolation of thymine-requiring mutant from streptomycin resistant strains

(i) Selection of minimum medium Selection of minimum medium had to be preceded in isolating thymine-requiring strains. Table 4 summarized

Table 4. Growth on various Minimum Medium with 11 Str^R Stains of *B. coagulans*

	Media	Str^R strains										
		1	2	3	4	5	6	7	8	9	10	11
A	liquid	−	−	−	−	−	−	−	−	−	−	−
	agar plate	−	−	−	−	−	−	−	−	−	−	−
B	liquid	+	+	+	+	+	+	+	+	+	+	+
	agar plate	−	−	+	+	+	+	+	+	+	+	+
C	liquid	+	+	+	+	+	+	+	+	+	+	+
	agar plate	+	+	+	+	+	+	+	+	+	+	+
D	liquid	+	+	+	+	+	+	+	+	+	+	+
	agar plate	+	+	+	+	+	+	+	+	+	+	+
E	liquid	+	+	+	+	+	+	+	+	+	+	+
	agar plate	+	+	+	+	+	+	+	+	+	+	+

Medium A: composed of glucose (5g/*l*), $MgSO_4$ (200 mg/*l*) and Spizizen salt soln.
Medium B: added casamino acid (5g/*l*) and tryptophane (50 mg/*l*) to the above medium A.
Medium C: added biotin (1 mg/*l*), thiamine (1 mg/*l*) and nicotinic acid (10 mg/*l*) to the above medium B
Medium D: added yeast extract (5 g/*l*) to the above medium B
Medium E: added yeast extract (5 g/*l*) to the above medium C

experimental results on various sorts of composition for selection of minimum medium. 11 streptomycin-resistant strains were used for selection of minimum medium. No colony on the minimum medium containing glucose, magnesium sulfate, and Spizizen salt solution (medium A) was observed. Minimum medium not containing any vitamins such as thiamine, d-biotin, and nicotinic acid, was observed to show changeable growth, depending upon properties of strains (Table 4, medium B). Since good growth on the medium containing glucose (5 g/*l*), magnesium sulfate (200 mg/*l*), Spizizen minimum salt solution, casamino acid (5 g/*l*), tryptophane (50 mg/*l*), biotin (1 mg/*l*), thiamine (1 mg/*l*) and nicotinic acid (10 mg/*l*) showed, these media (Table 2. MS Mmedium) were used throughout this study.

(ii) Isolation of thymine auxotrophic mutants Trimethoprim was, here, used a selective agent in obtaining spontaneous thymine-requiring mutants from streptomycin resistants of *Bacillus coagulans* ATCC 7050. 200 μg of thymine-requiring mutants per m*l* were first isolated, and then 10 colonies low thymine-requiring mutants (2 μg/m*l*) were isolated from 200 μg of thymine-requiring mutants. Fig. 2 shows procedures used here in obtaining low thymine-requiring mutants.

B. coagulans ATCC 7050 (parent strain)
↓
Str^R strain
↓
Precultured in PGY broth at 48 °C for 18 hrs
↓
Washed cells with Spizizen solution
↓
Cultured in MSM medium containing trimethoprim (10 μg/m*l*) and thymine (200 μg/m*l*)
↓ (if grown)
Plated on MSM medium containing trimethoprim and thymine
↓
Thimine-requiring mutant (200 μg/m*l*)
↓
Spreaded on MSM plate containing 2 μg of thymine/m*l*
↓
Thymine-requiring mutant (2 μg/m*l*)

Fig. 2. Procedure for Isolation of Thymine Auxotrophic Mutant

(iii) Test of cultivation with 2μg of thymine auxotrophic mutants per ml The isolated thymine-requiring (2 μg/m*l*) strains cultivated in MSM medium, showed slow growth as shown to take 23 hrs to reach logarithmic phase of these test strains under same conditions, comparing these with taking around 12 to 15 hours of other species of *Bacillus* (opinions of Seki *et al.*, unpublished). Cells were harvested for DNA extraction at optical density of 1.2 (Table 5) regarded as early exponential phase of these strains. [Methyl-^{3}H] thymine was added from the beginning of cultivation. Specific radioactivity of labeled DNAs of these strains was about 40,000 cpm per μg per m*l* as nearly same as those of other species of *Bacillus*, in usual, were.

Table 5. Growth of 2 μg/m*l* Thymine requiring Mutant of *B. coagulans* ATCC 7050 in MSM Broth Medium

Age (hrs)	Optical densitya
0	0.090
7.5	0.110
10.5	0.175
13.0	0.230
15.0	0.322
17.0	0.422
20.0	0.720
21.0	0.890
22.0	0.990
23.0	1.200

a Optical density was measured at 660 nm.

In this paper it could not be elucidated the mechanism why these strains would show slow growth, but these facts indicated that the growth rate of strains had no effect on preparing labeled DNA.

3. Procedures for hybridization

In a previous paper published here more recently, Seki *et al.* [4] estabilished the optimum conditions such as the amount of disk DNA (25 μg/m*l*) and input DNA (0.5 μg/m*l*), the determination of temperature and culturing time of the formation of DNA hybrid duplex. Most of procedures for hybridization were carried out according to their methods developed here as shown in details in Fig. 3, 4. In the procedures of preparing input DNA sol-

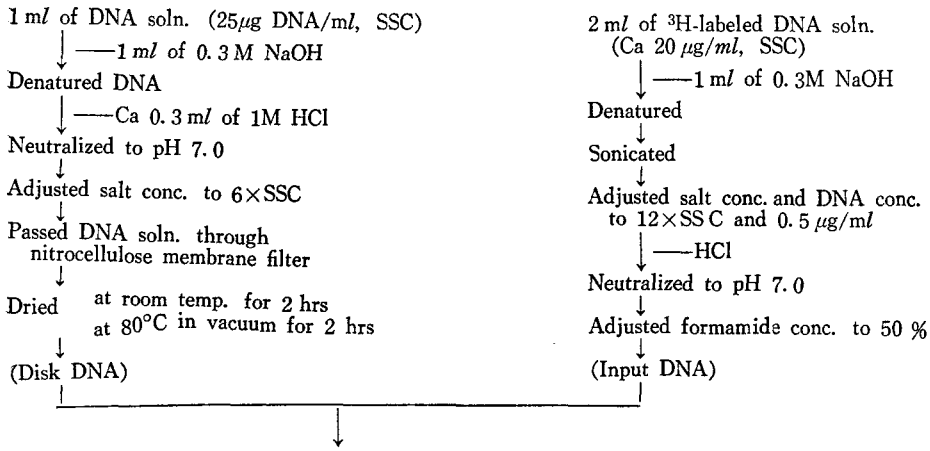


Fig. 3. Preparation for Disk DNA and input DNA

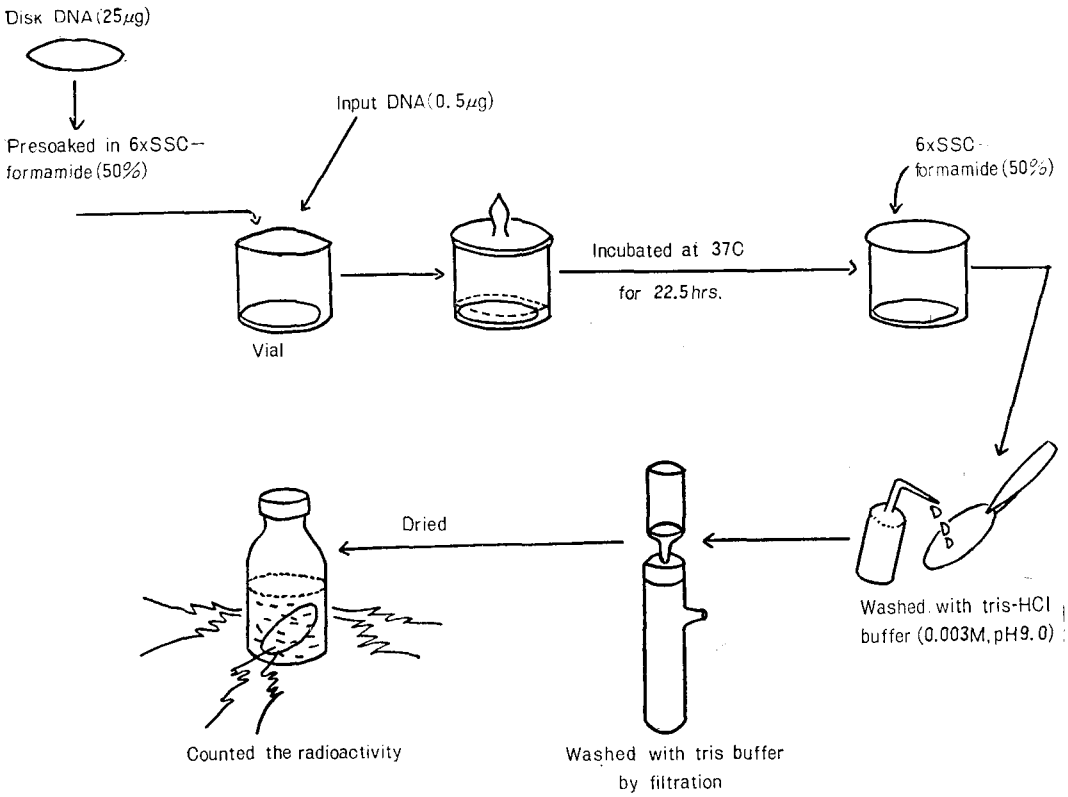


Fig. 4. Procedure for Hybridization

ution and adjusted to 50 % formamide solution just after all vials with caps were completely filled with the fixed disk DNA filters to avoid self-hybridization. During scintillation there were not found any diff erences depending upon the radioactive time ranged from one to five minute per vial. The unpaired DNAs were removed by, 4 times, washing with 3mM tris (hydroxymethyl)-aminomethane hydrochloride buffer solution (pH 9.0) after hybridization.

Table 6. DNA homologies among *B. coagulans* and between *B. coagulans* and other Species of *Bacillus*[a]

Disk DNA	Input DNA	
	B. coagulans ATCC 7050	*B. subtilis* 168
B. coagulans		
ATCC 7050	100%	3%
IFO 3886	90	4
IFO 3887	113	4
IFO 3557	98	4
IFO 12583	99	4
C-1	95	4
C-2	76	4
C-3	86	4
C-4	89	4
C-5	91	4
C-6	98	3
B. subtilis 168	4	100
B. licheniformis IFO 12107	4	8
B. pumilus IFO 12110	3	7
B. firmus ATCC 14575	2	3
B. lentus ATCC 10840	2	3
B. circulans ATCC 4513	2	—b
B. macelans ATCC 8244	2	—
B. polymyxa ATCC 842	2	—
B. sphaericus ATCC 14577	2	—
B. brevis ATCC 8246	3	—
B. brevis IFO 12334	2	—
B. laterosporus ATCC 64	2	—
B. pantothenticus ATCC 14576	2	—
Escherichia coli K-12	<1	<1

a Homology index is expressed as percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

b —, Not tested.

4. DNA homologies

Table 6 summarized the percent DNA-DNA hybridizations obtained by reacting ^{3}H-labeled DNA prepared from *B. coagulans* ATCC 7050 and *B. subtilis* 168 with unlabeled DNAs from other species of *Bacillus*. Among the different species of strains of *B. coagulans* used here, they were found to be closely related to the DNA of *B. coagulans* ATCC 7050 since the intraspecific DNA homology indexes among the 11 strains of *B. coagulans* using strain ATCC 7050 as the standard (^{3}H-labeled input DNA) showed 76 % or more respectively.

On the other hand, the interspecific DNA homologies among the 11 strain of *B. coagulans* using *B. subtilis* 168 as the standard (^{3}H-labeled input DNA) showed 4 to 3 % DNA homology indexes. The interspecific DNA homologies between *B. coagulans* ATCC 7050 as the standard (input DNA) and the type cultures of other species of *Bacillus* such as *B. subtilis* (168), *B. licheniformis* (IFO 12107), *B. pumilus* (IFO 12110), *B. firmus* (ATCC 14575), *B. lentus* (ATCC 10840), *B. circulans* (ATCC 4513), *B. macelans* (ATCC 8244), *B. polymyxa* (ATCC 842), *B. sphaericus* (ATCC 14577), *B. brevis* (ATCC 8246, IFO 12334), *B. laterosporus* (ATCC 64), and *B. pantothenticus*

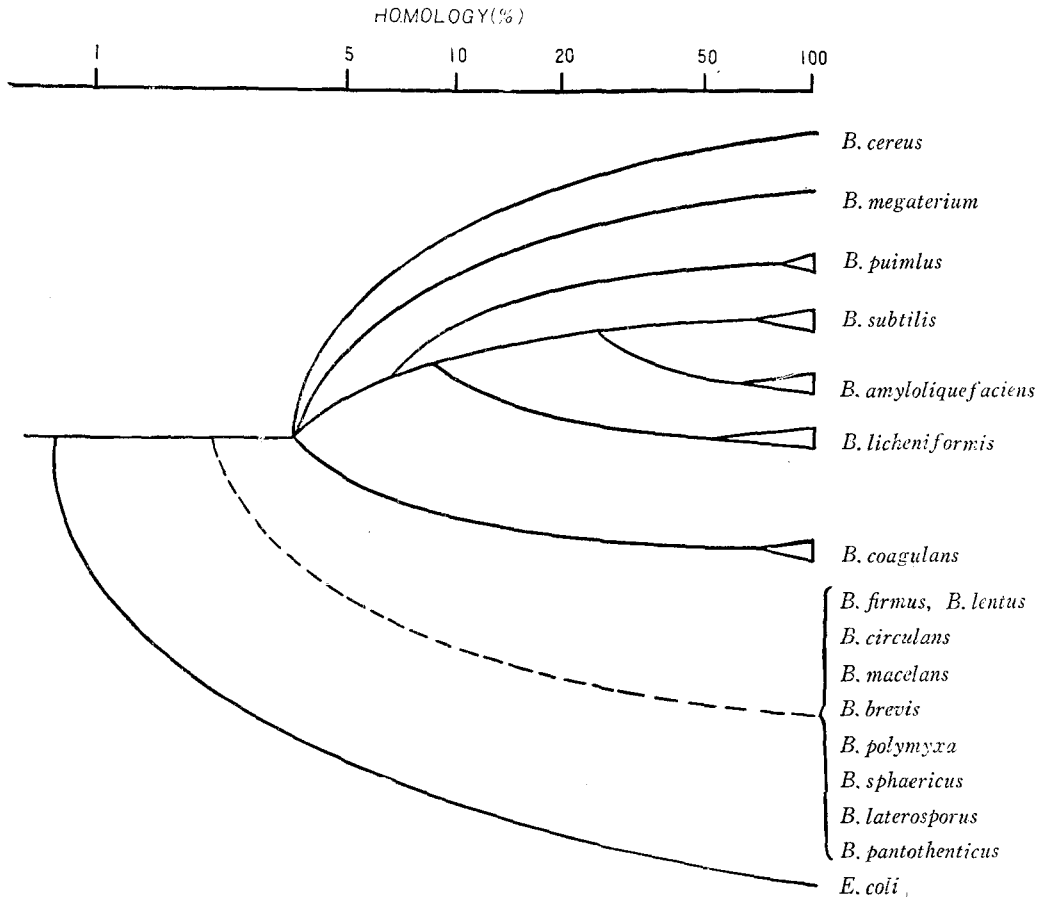


Fig. 5. Schematic Diagram of general Homology indexes among Genus *Bacillus*

(ATCC 14576) respectively, gave lower homology indexes of 4 to 2 %, while those of between *B. coagulans* ATCC 7050 or *B. subtilis* 168 and *Escherichia coli* K-12 were less than 1 %.

It was accepted that these data obtained here by DNA-DNA hybridization did not correlate the base composition to genetic DNA homologies between the DNAs of *B. coagulans* and the other species of Bacillus shown in Table 6, as it has generally been assumed that *E. coli* strains differed quite from *Bacillus* in the genetic structure of DNA sequence base.

Accordingly these findings indicated apparently that the newly isolated 6 strains and 5 strains obtained from the authentic sources fell into a group of strains of *B. coagulans*, because all 11 strains showed a high percentage of nucleotide sequence similarities. Particularly the results by DNA-DNA hybridization agreed excellently with those of the conventional taxonomic study according to the Bergey's Manual. As another result, it was confirmed that *B. coagulans* should be located on the line dividing the species of Group 1 and Group 2 (Smith & Gordon) because these strains did not belong to either Group 1 or Group 2 at molecular level.

Discussion

In summary, the most striking conclusion was the excellent agreement between the data obtained by DNA-DNA hybridization and those identified according to Bergey's Manual, although the results of several tests such as anaerobic growth, sodium azide reaction, NO_2 from NO_3 d-hydroxyacetone and deamination of phenylalanine etc. were not clear because of showing no growth in a specific media by the method of Gordon *et al.*

In present paper, it could not but be pointed out the relative importance of their nutritional features on the basis of the metabolic pathways so that strains of *Bacillus coagulans* be satisfactorily identified according to the conventional taxonomic methods, for reactions of "no growth" in the physiological tests were commonly represented. And the determnation of DNA homologies among these strains, based on hybridization of DNA on membrane filters, was an effective and highly reproducible procedures even in the second repeated experiments, it was accepted.

If more exhaustive studies of these strains involving some factors like GC contents, genome size, genetic characteristics (homo or hetero) of *B. coagulans* known to relate to produce lactic acid, should be made, it is thought that they will be a great milestone on the production of lactic acid in foreseeable future.

Acknowledgement

I wish to express my deepest gratitude to Prof. Y. Oshima, associate Prof. N. Takada, and Mr. T. Seki for their nice advices and continuous guidances throughout this study. I also desire to extend my heartist thank to all members of 4th laboratory who helped me in successfully carrying out this work. I would also like to express my hearty thankfulness to Prof. H. Taguchi and Mrs. F. Sawazumi for their heartfelt help during my studies here.

Finally I thank UNESCO, ICRO and Japanese Government for the fellowship granted during the International Post Graduate University Course in Microbiology 1975-1976 and the Department of Fermentation Technology, Osaka University for the facilities provided.

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서로 다른 11주의 *Bacillus coagulans* 와 13종의 *Bacillus* 속 14주를 deoxyribonucleic acid (DNA)-DNA hybridization method에 의해서 분류학적인 연구를 하였다.

사용한 *B. coagulans* 11주중 6주는 흙에서(일본 오사까교외) 분리했고, 나머지 5주는 ATCC, IFO에서 authentic strains을 얻어서 사용했다. 사용된 *B. coagulans*는 Bergey's Manual (8th ed)에 의거 Gordon氏들의 방법으로 동정한 결과 *B. coagulans*로서 확인되었다.

이렇게 동정된 *B. coagulans*을 분자 생물학적 차원에서 지금까지의 Conventional taxonomic study와의 관계를 연구하기 위해서 사용한 11주의 *B. coagulans* 중 ATCC 7070을 ^3H labeled input 즉 standard로 해서 사용했을때 *B. coagulans* 내의 intraspecific DNA homology indexes는 76% 이상으로 나타났다.

이와같은 발견은 Bergey's Manual에 의거한 conventional taxonomic study의 결과와 잘 일치하고 있었음으로 새로 분리한 6주와 authentic sources로부터 받은 5주는 같은 group의 *B. coagulans*라는 사실을 입증해 주었다. 그리고 *B. coagulans*와 다른 species의 *Bacillus* 속 즉 *B. pumilus* (168), *B. licheniformis* (IFO 12107), *B. pumilus* (IFO 12110), *B. firmus* (ATCC 14575), *B. lentus* (ATCC 10840), *B. circulans* (ATCC 4513), *B. macelans* (ATCC 8244), *B. polymyxa*, ATCC 842), *B. sphaericus* (ATCC 14577), *B. brevis* (ATCC 8246, IFO 12334), *B. laterosporus* (ATCC 64), *B. pantothenticus* (ATCC 14576) interspecific DNA homology indexes가 각각 2~4%을 보임으로써 *B. coagulans*는 molecular level면에서 이들 *Bacillus* 속과는 상동성(相同性) 관계가적음을 나타내었다. 반면에 *B. coagulans* (ATCC 7050)와 *E. coli* (F-12)와의 相同性은 1% 이하였다.

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