DEOXYRIBONUCLEIC ACID RELATEDNESS AMONG SOME SPORE-FORMING LACTIC ACID BACTERIA

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We studied the DNA base composition and DNA-DNA hybridization of 41 strains of spore-forming, lactic acid-producing bacteria including *Sporolactobacillus inulinus*. The DNA base composition of 41 strains studied ranged from 42.4 to 50.2 mol% guanine plus cytosine. The DNA of *S. inulinus* NRIC 1133^T showed extremely low homology (1 to 5%) to the strains of *Lactobacillus plantarum* and *Bacillus coagulans*, but it showed somewhat higher homology to “*Bacillus laevolacticus*,” “*Bacillus racemilacticus*,” “*Sporolactobacillus laevis*,” “*Sporolactobacillus laevis* var. *intermedius*,” and “*Sporolactobacillus racemicus*” (20–50%). Catalase-negative strains of spore-forming lactic acid bacteria, including the type strain of *S. inulinus*, showed relatively high homology values to one another and further divided into five groups and considered to be members of the genus *Sporolactobacillus*.

Sporolactobacillus inulinus is a catalase-negative, spore-forming, homo-fermenting lactic acid bacterium first reported by KITAHARA and SUZUKI(1). This species has characteristics intermediate between the genus *Bacillus* and the genus *Lactobacillus*(2).

We have reported on the morphological, biochemical, and physiological characteristics of spore-forming, lactic acid-producing bacteria including *S. inulinus* comparing with *Lactobacillus plantarum*, and *Bacillus coagulans*(2). The strains of those were determined by numerical analysis to be distinct from the type and authentic strains of *B. coagulans* and *L. plantarum*. The two large clusters were

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distinguished by catalase activity and production of acid from lactose and melibiose. However, their classification as spore-forming, lactic acid-producing bacteria was not definitive at the species level. Therefore, DNA-DNA homology studies are needed, not only for the classification at the species level, but also for the evaluation of phenotypic characteristics.

MILLER et al. (3) studied the DNA-DNA hybridization between the strains of *L. plantarum*, *S. inulinus*, and *B. coagulans* and reported that there were no cross reactions among these strains. In 1975, DELLAGHIO et al. (4) studied the DNA homologies among *Lactobacillus* species and reported that *S. inulinus* had a low homology index to any of the *Lactobacillus* species studied. Studies of 16S ribosomal RNA cataloguing by Fox et al. (5) showed that *S. inulinus* was distantly related to *Bacillus* species and to *Sporosarcina ureae*.

This paper deals with the DNA base composition and the DNA-DNA hybridization of representative spore-forming lactic acid bacteria selected from the strains used in the previous study (2).

MATERIALS AND METHODS

Bacterial strains. The strains used were *Sporolactobacillus inulinus* NRIC 1133^T (=ATCC 15538 =JCM 2257) (T=type strain), *S. inulinus* NRIC 1134 (S-50-C, from Prof. O. Kandler, University of Munich, West Germany), and 37 strains of the spore-forming lactic acid bacteria isolated by NAKAYAMA and YANOSHI (6, 7) and by AMEMIYA and NAKAYAMA (8). The strains used for comparison were: *Bacillus coagulans* NRIC 1005^T (=ATCC 7050 =JCM 2257), *B. coagulans* JCM 2258 (=IAM 1194), *Bacillus cereus* JCM 2152^T (=ATCC 14579), *Lactobacillus plantarum* NRIC 1067^T (=ATCC 14917), *L. plantarum* JCM 1158 (=ATCC 8041), *L. plantarum* JCM 1151 (=ATCC 10241), and *Lactobacillus yamanashiensis* subsp. *yamanashiensis* JCM 1153^T (=ATCC 27304). These *Lactobacillus* strains contain meso-diaminopimelic acid in the cell wall.

Isolation of DNA. Cells grown in GYP medium (2) at 30°C were harvested by centrifugation at the late exponential growth phase. Approximately 5 g of wet cells was used for DNA isolation by the method of SAITO and MIURA (9) with some modifications. Cells of *L. yamanashiensis* subsp. *yamanashiensis*, *L. plantarum*, and *B. cereus* were not lysed by lysozyme alone. In such cases, they were cultured in the medium supplemented with 1.5–5.0% glycine (10). The cell walls of these strains were lysed by incubation with N-acetyl muramidase SG (11) (Seikagaku Kogyo Co., Tokyo, Japan), Achromopeptidase crude (Wako Pure Chemicals Co., Osaka, Japan), and lysozyme (Seikagaku Kogyo Co.).

Determination of DNA base composition. The DNA base composition was calculated from the denaturation temperature of DNA (T_m) in SSC (SSC: 0.15 M NaCl and 15 mM trisodium citrate, pH 7.0) using the formula of MARMUR and DOTY (12). T_m was determined with a Beckman DU-8 spectrophotometer (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). Calf thymus DNA (Sigma

Chemical Co., St. Louis, Mo., U.S.A.) was used as a reference, containing 43.0 mol% guanine plus cytosine (G + C).

DNA-DNA hybridization. The SDS-membrane filter method described in previous reports (13, 14) was used for DNA-DNA hybridization. DNA was labeled with ^3H using a Nick Translation System, [^3H]- (New England Nuclear, Boston, Mass., U.S.A.). The hybridization reaction was carried out at 60°C for 40 hr. The radioactivity of the hybrid DNA was measured with a Beckman LS-7800 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

DNA base composition

Values of the DNA base composition are shown in Tables 1, 2, and 3. The DNA base composition of the strains of spore-forming lactic acid bacteria, including *S. inulinus* NRIC 1133^T, ranged from 42.4 to 50.2 mol% guanine plus cytosine (G + C). *B. coagulans* NRIC 1005^T and "*B. laevolacticus*" M-39 had higher percentages — 48.3 to 49.1 mol%. SUZUKI and KITAHARA (15), using paper chromatography, reported that the DNA base composition of the type strain of *S. inulinus* was 39.3 mol%. But MILLER et al. (16) found a higher percentage — 47.3 mol% G + C. Our results (47.0 mol% G + C) agreed with the value given by MILLER et al. (16).

DNA-DNA hybridization

Table 1 shows the results of DNA-DNA hybridization between *S. inulinus* NRIC 1133^T and strains in the genera *Bacillus* and *Lactobacillus*. When *S. inulinus*

Table 1. DNA-DNA homologies among strains of *Sporolactobacillus*, *Bacillus*, and *Lactobacillus*.

Strain	G + C content (mol%)	Labeled DNA from	
		<i>S. inulinus</i> NRIC 1133 ^T	<i>B. coagulans</i> NRIC 1005 ^T
<i>S. inulinus</i> NRIC 1133 ^T	47.0	100	6
<i>B. coagulans</i> NRIC 1005 ^T	49.1	3	100
JCM 2258	49.1	4	62
<i>B. cereus</i> JCM 2152 ^T	35.6	3	3
<i>L. plantarum</i> NRIC 1067 ^T	45.8	5	3
JCM 1551	45.5	2	2
JCM 1558	47.5	2	3
<i>L. yamanashiensis</i> subsp. <i>yamanashiensis</i>			
JCM 1153 ^T	37.2	1	3

Table 2. DNA-DNA homologies among strains of *Sporolactobacillus* and related organisms.

Strain	G+C content (mol%)	Labeled DNA from		
		<i>S. inulinus</i> NRIC 1133 ^T	" <i>S. laevas</i> " M-19	" <i>B. laevolacticus</i> " M-8
<i>S. inulinus</i>				
NRIC 1133 ^T	47.0	100	22	27
NRIC 1134	46.8	88	15	20
" <i>S. racemicus</i> "				
M-11	45.3	27	35	43
" <i>S. laevas</i> "				
M-18	42.4	22	101	30
M-19	46.2	22	100	33
M-87	44.5	32	23	56
" <i>S. laevas</i> var. <i>intermedius</i> "				
M-86	50.2	84	19	26
M-103	47.4	31	21	54
" <i>S. laevolacticus</i> "				
M-8	44.7	21	23	100
M-1	43.9	24	33	39
M-7	44.7	21	29	41
" <i>B. racemilacticus</i> "				
M-14	44.5	19	29	34
M-39	48.3	6	5	8
<i>B. coagulans</i>				
NRIC 1005 ^T	49.1	5	6	8
JCM 2258	49.1	7	5	6
<i>B. cereus</i>				
JCM 2152 ^T	35.6	6	6	7
<i>L. plantarum</i>				
NRIC 1067 ^T	45.8	4	4	6
<i>L. yamanashiensis</i> subsp. <i>yamanashiensis</i>				
JCM 1153 ^T	37.2	4	5	5
Calf thymus	43.0	0	1	2

NRIC 1133^T was used as a reference, the homology indexes to four strains of the *Lactobacillus* species ranged from 1 to 5% and from 3 to 4% to three strains of the *Bacillus* species. When *B. coagulans* NRIC 1005^T was used as a reference, the homology indexes were 6% to *S. inulinus* NRIC 1133^T and from 2 to 3% to four strains of the *Lactobacillus* species and another *Bacillus* species. These results indicate that there is little DNA homology between *S. inulinus* and either *B. coagulans* or *L. plantarum*.

Table 2 shows the results of DNA-DNA hybridization of the strains of *S. inulinus* with some strains of the "*B. laevolacticus*," "*B. racemilacticus*," "*S. laevas*," "*S. laevas* var. *intermedius*," "*S. racemicus*." *S. inulinus* NRIC 1134 and "*S. laevas* var. *intermedius*" M-86 were highly homologous to *S. inulinus* NRIC 1133^T. "*S. laevas*" M-19 was highly homologous to "*S. laevas*" M-18, and only slightly homologous to "*S. laevas*" M-87. The reference strains used here showed moderately higher homology values to the strains of *S. inulinus* (NRIC 1133^T and NRIC 1134), "*S. laevas*" (M-18, M-19, and M-87), "*S. laevas* var. *intermedius*" (M-103), "*S. racemicus*" (M-11), "*B. laevolacticus*" (M-1, M-7, and M-8), "*B. racemilacticus*" (M-14), and low values to the strains of *L. plantarum* and *L. yamanashiensis* subsp. *yamanashiensis*, *B. coagulans*, and *B. cereus*. These strains, except "*B. racemilacticus*" M-39, are considered to be closer to *S. inulinus* than to the *Bacillus* and *Lactobacillus* species. "*B. racemilacticus*" M-39 showed extremely low homology values to the three references. This strain was identified as *B. coagulans* in a previous paper (2).

Table 3 shows the results of DNA-DNA hybridization among the strains of *S. inulinus*, "*S. laevas*," "*S. laevas* var. *intermedius*," and "*S. racemicus*." The DNA homology divided them into five groups. The strains in these groups are discussed below in connection with their phenotypic characteristics (2).

Group 1: This group comprises the three strains, *S. inulinus* NRIC 1133^T, *S. inulinus* NRIC 1134, and "*S. laevas* var. *intermedius*" M-86. These strains showed extremely high homology to each other, and "*S. laevas* var. *intermedius*" M-86 was identified as *S. inulinus*. This group is characterized by an inability to produce acid from galactose, lactose, and melibiose (2).

Group 2: This group comprises fifteen strains of "*S. laevas*" (12 strains) and "*S. laevas* var. *intermedius*" (3 strains). When "*S. laevas*" strains (M-114, M-88) were used as references, the homology indexes within the strains of this group ranged from 67 to 101%. The group is characterized by an inability to produce acid from inulin, lactose, and melibiose, and a tolerance of high concentrations of NaCl (2).

Group 3: This group comprises nine strains of "*S. racemicus*" (7 strains) and "*S. laevas* var. *intermedius*" (2 strains). When "*S. racemicus*" M-116 was used as a reference, the homology indexes ranged from 55 to 119%. This group is characterized by an ability to produce acid from inulin and a sensitivity to NaCl (2). Furthermore, the strains do not produce acid from lactose and melibiose.

Group 4: This group comprises four strains. "*S. racemicus*" M-13 and "*S. laevas* var. *intermedius*" M-98 showed a high homology index (91% and 84% respectively) to the reference strain "*S. racemicus*" M-17. "*S. laevas* var. *intermedius*" M-96 showed a high homology index (79%) to the reference strain "*S. laevas* var. *intermedius*" M-98. The group is characterized by an ability to produce acid from melibiose, lactose, and inulin (2).

Group 5: This group comprises the two strains, "*S. laevas*" M-18 and "*S. laevas*" M-19. These organisms showed an extremely high homology index of 101%

Table 3. DNA homologies among the strains of

Homology group	Strain	Labeled			
		G + C content (mol%)	<i>S. inulinus</i> NRIC 1133 ^T	<i>"S. laevas"</i> M-114	
1	<i>S. inulinus</i> NRIC 1133 ^T	47.0	100	100	25
	<i>S. inulinus</i> NRIC 1134	46.8	88		
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M- 86	50.2	88		35
2	<i>"S. laevas"</i> M- 45	45.3	32		75
	<i>"S. laevas"</i> M- 46	45.5	41		76
	<i>"S. laevas"</i> M- 50	45.2	41		81
	<i>"S. laevas"</i> M- 51	42.5	34		70
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M- 54	45.4	42		72
	<i>"S. laevas"</i> M- 56	45.0	38		73
	<i>"S. laevas"</i> M- 84	46.2	47		101
	<i>"S. laevas"</i> M- 87	44.5	33		80
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M-103	47.4	33		78
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M-113	45.3	43		86
	<i>"S. laevas"</i> M-114	44.8	37	33	100
	<i>"S. laevas"</i> M- 47	44.7		38	
	<i>"S. laevas"</i> M- 59	44.7		33	
3	<i>"S. laevas"</i> M- 88	46.7		34	
	<i>"S. laevas"</i> M-101	43.4		41	
	<i>"S. racemicus"</i> M- 10	43.6		22	
	<i>"S. racemicus"</i> M- 16	42.9		25	
	<i>"S. racemicus"</i> M- 32	45.9		25	
	<i>"S. racemicus"</i> M- 33	46.3		17	
	<i>"S. racemicus"</i> M- 61	44.9		25	
	<i>"S. racemicus"</i> M-115	45.8		39	
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M- 85	46.2	40		54
4	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M- 99	45.4	35		48
	<i>"S. racemicus"</i> M-116	44.4	42		53
	<i>"S. racemicus"</i> M- 13	45.8		22	
	<i>"S. racemicus"</i> M- 17	45.0		22	
	<i>"S. laevas</i> var. <i>intermedius"</i>				
	M- 96	42.5	32		58
	<i>"S. laevas</i> var. <i>intermedius"</i>				
5	M- 98	44.8	26	32	57
	<i>"S. laevas"</i> M- 18	43.0	24	23	24
	<i>"S. laevas"</i> M- 19	43.0	24	16	28
Calf thymus			0	0	1

5 groups of spore-forming lactic acid bacteria.

DNA from

" <i>S. laevas</i> " M-88	" <i>S. racemicus</i> " M-116	" <i>S. racemicus</i> " M-17	" <i>S. laevas</i> " M-19	" <i>S. laevas</i> var. <i>intermedius</i> " M-98
24	22	24	22 15	26
		27	19	
67	38	47		62 61 64 58 59 59 66 50
82	52	60	23 21	55 67 55
84 72 77 100 93	46 42 51 35 48	61 61 64 61 67		
31 49 33 33 49 48	56 55 64 64 72 119	39 40 43 30 46 38		
35	91	38		45
33 36	63 100	41 34		47 49
49 64	28 37	91 100		
				79
64	41	84		100
26 27	19 20	29 25	101 100	27 26
4	5	4	1	0

and they showed extremely low homology values to other reference strains.

The strains in these five groups of *Sporolactobacillus* may be separated into species-presuming groups homogeneous in phenotypic characteristics (2) and DNA-DNA relatedness.

Isoprenoid quinones and cellular fatty acid composition are considered to be useful for bacterial taxonomy. UCHIDA and MOGI(17) examined the fatty acid composition of *S. inulinus*, "*B. laevolacticus*," "*B. racemilacticus*," "*S. laevis*," "*S. racemicus*," and *B. coagulans*, and found "iso-anteiso-type" of fatty acids in these species, while "unsaturated-type" fatty acids were found in *L. plantarum*, *L. yamanashiensis*, and *L. casei*. Other *Lactobacillus* species (18, 19) and the *Clostridium* species (20) also had "unsaturated-type" fatty acids.

COLLINS and JONES (21) studied the menaquinone systems of the strains of *S. inulinus*, "*B. laevolacticus*," "*S. laevis*," "*S. racemicus*," and *B. coagulans*, and found menaquinone with seven-isoprenoid units (MK-7) as a major isoprenoid quinone in these species. These facts indicate that *Sporolactobacillus* strains are quite different from the strains of *Lactobacillus* species, and are rather closely related to the genus *Bacillus*. However, they were distantly related even to the strains of *B. coagulans*, which is considered to be the closest species to *Sporolactobacillus* among the *Bacillus* species (17). The *Sporolactobacillus* strains used in the present study showed moderately high homology to each other.

The present study concludes that the strains named *Sporolactobacillus* form an independent taxon from the genera *Bacillus* and *Lactobacillus*. *Sporolactobacillus* is likely to be treated as an independent genus belonging to the family *Bacillaceae* as in Bergey's Manual of Determinative Bacteriology, 8th edition (22).

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