Deoxyribonucleic Acid Relatedness of *Bacillus circulans* Jordan 1890 Strains

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Deoxyribonucleic acid reassociation measurements revealed that 50 of 111 *Bacillus circulans* Jordan 1890 strains examined could be separated into 10 groups of genetically related organisms. The results of deoxyribonucleic acid relatedness studies and phenotypic comparisons suggested that five of these groups were hitherto undescribed species and that four others probably represented previously, but inadequately, described species. The tenth group, which included the type strain, represented the species *B. circulans* sensu stricto. Our data show that *B. circulans* and *Bacillus macerans* Schardinger 1905 are genetically and phenotypically distinct species. Thus, the phenotypic heterogeneity of the species *B. circulans* sensu lato is not due to inherent variability of genetically related strains but rather is due to inclusion of genetically unrelated organisms in this taxon.

Jordan described the species *Bacillus circulans* in 1890 (8). In 1916, Ford isolated a morphologically dissimilar strain but classified it as *B. circulans* because, like the strain of Jordan, its colonies also displayed internal circular movement (4). The strain of Jordan has been lost. Because strain 26 of Ford is the only historical strain available, it has been designated as the type strain.

Continued studies by other researchers have disclosed a morphologically, nutritionally, physiologically, immunologically, and chemically heterogeneous group (1, 6, 7, 10, 12, 13). Our studies have established that of 123 strains identified as *B. circulans*, only 17 have guanine-plus-cytosine (G+C) contents (37 to 40 mol%) like the type strain. Deoxyribonucleic acid (DNA) reassociation experiments have revealed that 10 of these 17 strains are closely related to each other and to the type strain (9). The present study was undertaken to determine by DNA reassociation experiments the taxonomic position of the strains misclassified as *B. circulans*. Our data suggest that at least five hitherto undescribed species and four previously, but inadequately, described species may be misclassified as *B. circulans*.

MATERIALS AND METHODS

Bacterial strains. The *B. circulans* strains used in this study have been listed previously (9). Also included in this study were strains NRRL NRS-18, NRRL NRS-609, NRRL NRS-888, and NRRL NRS-1105, the type strains of *Bacillus stearothermophilus* Donk 1920, *Bacillus coagulans* Hammer 1915, *Bacillus macerans* Schardinger 1905, and *Bacillus polymyxa* (Prazmowski) Mace 1889, respectively.

Characterization. The strains were characterized as

described by Nakamura and Swezey (9). In this study we also tested for urease activity by the method of Edwards and Ewing (3).

DNA isolation, G+C ratios, and DNA reassociation.

The procedures used for DNA isolation and final purification by hydroxyapatite chromatography and for estimation of DNA reassociation values by spectrophotometric measurement of renaturation rates have been described previously (9). The equation of De Ley et al. (2) was used to calculate the reassociation values (percentages). All determinations of DNA reassociation values were repeated twice.

To determine the G+C content, the buoyant density of a DNA was measured by CsCl density gradient centrifugation; the internal standard which we used was *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA purchased from Sigma Chemical Co., St. Louis, Mo. (11).

RESULTS

Because a large number of strains were involved, DNA reassociation values were determined only among organisms segregated according to similarities in G+C contents or phenotypic characteristics or both. Such determinations established that 50 of the 111 strains identified as *B. circulans* could be separated into 10 distinct DNA relatedness groups (Table 1), each consisting of a reference strain and organisms genetically related to it. Experiments showed that the extent of DNA reassociation among members of any one group was high (70 to 100%). However, intergroup DNA reassociation values were consistently low (35% or less). The largest group (group 1) consisted of nine organisms and the type strain of *B. circulans* (strain NRRL NRS-726), and these organisms had G+C contents of 37.4 to 38.9 mol%. The remaining nine groups (groups 2 through 10)

TABLE 1. DNA relatedness of selected *B. circulans* strains

Group or species	Strain (NRRL no.)	G+C content (mol%)	% Reassociation with DNA from strain: ^a									
			NRRL NRS- 726 ^{Tb}	NRRL NRS- 1434	NRRL NRS- 1347	NRRL NRS- 1346	NRRL BD- 537	NRRL NRS- 1173	NRRL NRS- 381	NRRL NRS- 666	NRRL NRS- 838a	NRRL NRS- 290
1 ^c		37.4–38.9 ^d	63–100 ^d									
2	NRS-1434	45.6	15	(100) ^e								
	NRS-1435	45.6	28	81								
	NRS-1436	46.2	21	94								
3	NRS-1347	47.5	18	26	(100)							
	NRS-1348	49.1	ND ^f	20	76							
	NRS-1350	47.3	20	22	85							
4	NRS-1346	47.1	10	2	35	(100)						
	NRS-1349	48.5	9	7	18	98						
	NRS-1351	47.0	ND	2	26	100						
5	B-745	48.5	ND	34	10	24	98					
	B-1373	47.8	ND	20	6	21	93					
	B-1488	48.1	16	22	9	19	89					
	BD-536	50.0	ND	18	14	25	100					
	BD-537	48.7	ND	9	10	29	(100)					
	NRS-924	49.9	14	20	9	20	78					
6	NRS-1173	48.4	ND	34	22	28	6	(100)				
	NRS-1174	49.5	12	ND	19	20	9	86				
	NRS-1175	47.6	ND	16	26	22	12	100				
	NRS-1177	49.5	12	23	24	17	10	84				
	NRS-1343	50.2	ND	ND	17	25	5	71				
	NRS-1356	47.8	ND	11	26	18	10	77				
7	NRS-381	51.4	2	25	27	24	10	15	(100)			
	NRS-382	51.7	ND	11	ND	ND	4	20	94			
	NRS-383	50.9	ND	ND	32	ND	ND	18	92			
	NRS-384	51.8	ND	15	ND	7	6	ND	100			
	NRS-386	50.3	8	ND	25	20	ND	ND	100			
8	NRS-666	51.5	2	3	26	10	8	10	25	(100)		
	NRS-676	51.2	ND	12	20	3	9	14	27	75		
	NRS-678	50.2	2	ND	ND	17	ND	ND	26	70		
	NRS-679	50.9	ND	10	13	3	12	20	20	84		
	NRS-1171	51.5	ND	ND	ND	ND	ND	ND	30	100		
9	NRS-625	53.6	9	2	10	8	14	14	10	16	79	
	NRS-639	54.1	ND	ND	ND	11	ND	12	12	10	100	
	NRS-838a	54.4	8	16	12	ND	8	ND	18	20	(100)	
	NRS-839a	53.3	ND	ND	15	ND	ND	19	15	23	98	
	NRS-1000	53.9	3	12	ND	18	13	ND	17	14	100	

10	B-142	52.7	9	2	ND	ND	16	2	20	5	89
	NRS-290	52.7	ND	ND	10	12	21	10	18	10	(100)
	NRS-359	52.6	14	16	19	17	ND	5	11	9	100
	NRS-1136	52.7	ND	ND	ND	ND	ND	8	29	10	100
	<i>B. polymyxa</i> NRS-1005 ^T	43-46	21	18	30	20	24	10	16	18	26
	<i>B. coagulans</i> NRS-609 ^T	47-48	20	20	29	25	30	10	15	27	20
	<i>B. macerans</i> NRS-888 ^T	49-51	10	18	12	2	34	4	14	28	14
	<i>B. stearothermophilus</i>	49-53	14	8	8	10	17	22	25	25	29
	NRS-18 ^T										

^a Reassociation values are averages of two determinations.

^b T = type strain.

^c Group 1 included strains NRRL NRS-313, NRRL NRS-358, NRRL NRS-726^T, NRRL NRS-727, NRRL NRS-728, NRRL NRS-729, NRRL NRS-746, NRRL NRS-765, NRRL NRS-831, and NRRL NRS-1108.

^d Data from reference 9. The mean value of reassociation with strain NRRL NRS-726^T DNA for nine strains was 94%.

^e The values in parentheses indicate that by definition the reassociation value was 100%.

^f ND, Not done.

contained from three to six members. The strains in groups 2 through 6 had G+C contents that ranged from 45.6 to 50.2 mol%. In groups 7 through 10, the G+C contents ranged from 50.3 to 54.4 mol%. DNAs with G+C contents that range from 45 to 54 mol% have been found in *B. coagulans*, *B. macerans*, *B. polymyxa*, and *B. stearothermophilus* (5). Our low DNA relatedness values suggested that none of the nine groups was related genetically to these four established species (Table 1).

Table 2 shows a comparison of the phenotypic characteristics of the 10 DNA relatedness groups. In general, whereas the groups showed pronounced phenotypic heterogeneity, individuals within groups were highly homogeneous. All of the groups were catalase positive and were negative for acetylmethylcarbinol production (Voges-Proskauer [VP] test), oxidase, egg yolk lecithinase, H₂S production, and growth at 55°C or higher. Except for group 7, all of the groups were active carbohydrate fermenters.

Group 11 consisted of 61 strains that gave low DNA reassociation values (30% or less) with the reference strains of the first 10 groups. The G+C contents of these strains ranged from 43 to 55 mol%. The characteristics of group 11 are summarized in Table 2. This group was highly heterogeneous, a condition that appropriately describes the species *B. circulans* in its present state.

DISCUSSION

On the basis of DNA reassociation studies, 10 distinct groups were identified among 111 strains identified as *B. circulans*. As reported previously (9), because the major group (group 1) contains the type strain, this group represents the species *B. circulans* sensu stricto. According to Table 2, this *B. circulans* group is phenotypically distinct from the other nine DNA relatedness groups. Furthermore, in contrast to the statement in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (5), *B. circulans* is distinguishable from *B. macerans* by differences in the following characteristics: growth in 5% NaCl, utilization of citrate, reduction of nitrate to nitrite, and G+C content (Table 3). The level of DNA relatedness between the two species is low (Table 1).

Ranging from 45 to 54 mol%, the G+C contents of groups 2 through 10 encompass the range of base contents of DNAs from a group of known species which includes *B. coagulans* Hammer 1915, *B. macerans* Schardinger 1905, *B. polymyxa* (Prazmowski) Macer 1889, and *B. stearothermophilus* Donk 1920. However, low DNA relatedness values indicate that none of these nine groups is related to these four established species.

TABLE 2. Characteristics of the DNA relatedness groups

Characteristic	Group ^a										
	1 (n = 10) ^b	2 (n = 3)	3 (n = 3)	4 (n = 3)	5 (n = 6)	6 (n = 6)	7 (n = 5)	8 (n = 5)	9 (n = 5)	10 (n = 4)	11 (n = 61)
Growth under anaerobic conditions	10 ^c	3	0	0	6	6	0	5	5	5	ND ^d
Growth in 3% NaCl	10	0	0	1	5	4	0	5	0	4	29
Growth in 5% NaCl	10	0	0	0	2	0	0	5	0	1	12
Growth in 7% NaCl	7	0	0	0	0	0	0	0	0	0	3
Growth at pH 5.6	10	3	3	3	6	4	4	0	5	4	40
Growth with lysozyme	0	0	3	0	0	0	5	5	0	4	15
Growth at 55°C	0	0	0	0	0	0	0	0	0	0	ND
Hydrolysis of:											
Casein	0	3	0	1	5	0	0	0	0	0	17
Starch	10	0	3	3	6	6	0	5	5	4	39
Tween 80	9	3	3	3	0	2	1	0	5	0	26
Nitrate reduced to nitrite	0	0	0	0	0	0	0	5	0	4	28
VP test	0	0	0	0	0	0	0	0	0	0	6
Urease activity	0	3	2	3	0	6	0	5	5	1	36
Egg yolk lecithinase	0	0	0	0	0	0	0	0	0	0	3
H ₂ S production	0	0	0	0	0	0	0	0	0	0	2
Catalase	10	3	3	3	6	6	5	5	5	4	61
Oxidase	0	0	0	0	0	0	0	0	0	0	2
pH in VP broth < 5.5	10	0	0	0	5	6	0	5	0	4	40
Indole	0	0	0	0	0	0	0	0	0	0	1
Utilization of:											
Acetate	10	3	3	2	6	6	5	5	5	2	49
Citrate	0	0	0	0	0	0	0	0	5	0	20
Fumarate	10	3	3	0	0	0	5	0	5	0	24
Malate	10	3	3	2	0	0	5	0	5	0	25
Succinate	1	3	3	0	0	0	5	0	5	0	21
Fermentation of:											
Arabinose	10	3	3	3	6	6	0	5	5	4	53
Cellobiose	10	3	3	3	6	6	0	5	5	4	59
Fructose	10	3	3	3	6	6	0	5	5	4	61
Galactose	10	3	3	3	6	6	5	5	5	4	59
Glucose	10	3	3	3	6	6	5	5	5	4	61
Lactose	10	3	3	3	6	6	5	5	5	4	55
Maltose	10	3	3	3	6	6	5	5	5	4	61
Mannitol	10	3	3	3	6	6	5	5	5	4	53
Mannose	10	3	3	3	6	6	0	5	5	4	61
Melibiose	10	3	3	3	6	6	0	5	5	4	56
Rhamnose	10	3	3	3	6	6	5	5	5	1	41
Ribose	10	3	3	3	6	6	5	5	5	4	59
Salicin	10	3	3	3	6	6	5	5	5	4	59
Sorbitol	10	3	0	0	1	2	0	0	5	0	21
Sucrose	10	3	3	3	6	6	5	5	5	4	61
Trehalose	10	3	3	3	6	6	5	5	5	4	61
Xylose	10	3	3	3	6	6	5	5	5	4	58

^a Groups 1 through 10 were composed of organisms showing DNA relatedness. Group 11 contained all strains not included in the 10 groups of related strains.

^b Number of strains in the group.

^c Number of positive strains.

^d ND, Not done.

Phenotypically, the inability of the members of the groups to grow at 55°C eliminates classification as either *B. coagulans* or *B. stearothermophilus* (Table 3). Furthermore, in contrast to

groups 2 through 10, *B. coagulans* gives a positive VP test, and *B. stearothermophilus* does not ferment arabinose, mannitol, or xylose.

Because groups 2 through 10 are VP test

TABLE 3. Characteristics for differentiating groups 1 through 10 from *B. coagulans*, *B. macerans*, *B. polymyxa*, and *B. stearothermophilus*

Characteristic	Group										<i>B. coagu-</i> <i>lans</i> ^a	<i>B. mace-</i> <i>rans</i> ^a	<i>B. poly-</i> <i>myxa</i> ^a	<i>B. stearo-</i> <i>thermo-</i> <i>philus</i> ^a
	1	2	3	4	5	6	7	8	9	10				
Growth in:														
5% NaCl	+	—	—	—	v	—	—	+	—	v	—	—	—	+
7% NaCl	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Growth at pH 5.6	+	+	+	+	+	v	v	—	+	+	+	+	+	—
Growth with lysozyme	—	—	+	—	—	—	+	+	—	+	—	v	—	—
Growth under anaerobic conditions	+	+	—	—	+	+	—	+	+	+	+	+	+	—
Growth at 55°C	—	—	—	—	—	—	—	—	—	—	+	—	—	+
Hydrolysis of:														
Starch	+	—	+	+	+	+	—	+	+	+	+	+	+	+
Casein	—	+	—	v	v	—	—	—	—	—	+	—	+	v
VP test	—	—	—	—	—	—	—	—	—	—	+	—	+	—
Nitrate reduced to nitrite	—	—	—	—	—	—	—	+	—	+	v	+	+	v
Citrate utilization	—	v	—	—	—	—	—	—	+	—	v	+	—	—
Fermentation of:														
Glucose	+	+	+	+	+	+	+	+	+	+	+	⊕	⊕	+
Arabinose	+	+	+	+	+	+	—	+	+	+	v	⊕	⊕	—
Xylose	+	+	+	+	+	+	+	+	+	+	v	⊕	⊕	—
Mannitol	+	+	+	+	+	+	+	+	+	+	v	⊕	⊕	—
G+C content (mol%)	37–39	45–46	47–49	47–48	47–50	47–50	50–52	50–52	53–54	50–53	47–48	49–51	43–46	49–53

^a Data from reference 5.^b +, Positive; —, negative; v, variable; ⊕, positive with gas production.

negative and largely negative for citrate utilization, reduction of nitrate to nitrite, and caseinase activity, they are probably not related to *B. polymyxa*, which is VP test positive, reduces nitrate to nitrite, and hydrolyzes casein (Table 3). Groups 8 and 10 reduce nitrate to nitrite but do not hydrolyze casein. Groups 2 and 5 hydrolyze casein but do not reduce nitrate.

B. macerans is VP test negative, reduces nitrate to nitrite, and utilizes citrate. Since they are negative for all three of these tests, groups 2 through 7 are most likely not related to *B. macerans* (Table 3). Although groups 8 and 10 reduce nitrate to nitrite, their ability to grow in lysozyme and their failure to utilize citrate differentiate them from *B. macerans*. Group 9 differs from *B. macerans* in not being able to reduce nitrate to nitrite.

DNA relatedness measurements and phenotypic studies have revealed that the majority of the strains which we examined have been erroneously classified as *B. circulans*. According to our results, among the 111 erroneously classified organisms there were nine genetically unrelated groups (hence, nine distinct species). Members of five of these nine groups may represent species that have not been described previously, and some of the strains in four of the groups were originally classified as species other than *B. circulans*. For example, group 5 includes strain NRRL NRS-924, which was received as "*Bacillus pabuli*" Schieblisch 1923. Group 8 contains strain NRRL NRS-666, which was received as "*Bacillus lautus*" Batchelor 1919. Included in group 9 are strains NRRL NRS-625 and NRRL NRS-626, which were received as "*Bacillus nitidus*" Bredemann and Heigener 1935; strains NRRL NRS-639 and NRRL NRS-1000, which were received as "*Bacillus validus*" Bredemann and Heigener 1935; and strains NRRL NRS-838a and NRRL NRS-839a, which were contaminants of a culture identified as "*Bacillus cohaerens*" Meyer and Gottheil 1901. Strain NRRL NRS-290 in group 10 was received as "*Bacillus amylolyticus*" Kellerman and MacBeth 1912. The above observations suggest that group 5 could be classified as "*B. pabuli*", group 8 could be classified as "*B. lautus*", group 9 could be classified as "*B. nitidus*" or "*B. validus*", and group 10 could be classified as "*B. amylolyticus*".

Also present were 61 strains which were not

genetically related to groups 1 through 10 and displayed widely varying characteristics. Consequently, the phenotypic heterogeneity of *B. circulans* is not due to the inherent variability of genetically related strains but is derived from the inclusion of a number of unrelated organisms in this taxon.

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