Deoxyribonucleic Acid Homologies of *Lactobacillus amylophilus* and Other Homofermentative Species

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The deoxyribonucleic acid (DNA) relatedness between a newly described, homofermentative species, *Lactobacillus amylophilus*, and a broad spectrum of homofermentative lactobacilli was determined. A low range of DNA reassociation levels (5 to 33%) was observed between *L. amylophilus* and those species (*L. amylovorus*, *L. bulgaricus*, *L. casei*, *L. coryniformis*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*, and *L. xylosus*) with which it shared one or more phenotypic characteristics (such as the production of mainly L-(+)-lactic acid from glucose, hydrolysis of starch, the sugar fermentation pattern) or which had a guanine plus cytosine content of 45 mol%. A lack of DNA relatedness (6 to 20% reassociation) was noted between *L. amylophilus* and 10 other homofermentative lactobacilli. A DNA reassociation of 90 to 98% was measured among the tested strains of *L. amylophilus*. Purified cell walls of *L. amylophilus* contained alanine, aspartic acid, glutamic acid, and lysine, but no diaminopimelic acid. The low DNA relatedness indicated that the *L. amylophilus* strains were not highly related genetically to the established homofermentative species and, hence, that they represent strains of a distinct genospecies.

Earlier, we described a new species, *Lactobacillus amylophilus*, that hydrolyzed starch and produced mainly L-(+)-lactic acid homofermentatively (14). The establishment of the new species was based on phenotypic characterization only. To confirm that the organism was a genotypic as well as a phenotypic species and to investigate its possible relationship to lactobacilli with similar characteristics, deoxyribonucleic acid (DNA) reassociation studies were performed.

The new species, of which substantial numbers of cells were present in all swine waste-corn fermentations examined, elaborated extracellular amylolytic enzymes to facilitate starch metabolism. The specific epithet, *amylophilus*, was given to indicate the capacity to ferment starch. Because the name *Lactobacillus amylophilus* Nakamura and Crowell was not included on the Approved Lists of Bacterial Names (19), it was not considered to be validly published as of 1 January 1980. However, the name has subsequently been validated by being announced in the *International Journal of Systematic Bacteriology* (13).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The strains were provided by the Agricultural Research Culture Collection maintained at the Northern Regional Research Center. Strains NRRL B-4538, B-4539, B-4540, and B-4542 are strains of another recently described starch-hydrolyzing species, *L. amylovorus*, isolated from several

different cattle waste-corn fermentations (12). The sources of the *L. amylophilus* strains also were several different swine waste-corn fermentations. Working stocks were grown for 24 to 48 h in MRS agar (5) slabs at appropriate temperatures and stored at 4°C. For DNA extraction and cell wall preparation, cultures were grown in MRS broth.

DNA purification and renaturation. DNA was extracted and purified by a modification of Marmur's method (10). The modification involved the use of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) chromatography (9) to produce highly purified DNA preparations. The purity and quality of each DNA preparation were checked by monitoring the absorbancy ratio at 260/280 nm (1.8 to 1.9) and at 260/230 nm (2.0 to 2.3) as described by Marmur (10) and by melting (T_m) curves, which include hyperchromicity values from 38 to 40% (8).

In preparation for renaturation studies, DNA was sheared by two passages through a French pressure cell at 10,000 lb/in². After filtration through a 0.45- μ m membrane (Millipore Corp.), the sheared DNA solution was dialyzed exhaustively against a solution containing 5 ml of SSC (0.1 M NaCl-0.015 M trisodium citrate, pH 7.0) per liter of 0.001 M ethylenediaminetetraacetate. After dialysis, the DNA solution was dried by freeze-drying. The dried material was dissolved in 2 ml of double-distilled water.

The kinetics of DNA renaturation were measured in reaction mixtures that contained 0.3 ml of 10 $\times$ SSC, 0.2 ml of dimethyl sulfoxide, approximately 75 μ g of DNA, and double-distilled water to bring the volume to 1 ml. In reaction mixtures containing two different DNAs, the amounts (about 37.5 μ g) of the nucleic acids were adjusted to differ by no more than 0.15 μ g. The reaction mixtures were placed in 0.4-ml quartz cuvettes that were sealed with Teflon-lined stoppers. Denaturation and renaturation were effected and mon-

TABLE 1. List of strains used in this study.

Serial no.	Laboratory no.	Received as:		Source
		Name	Strain	
1	NRRL B-4495 ^a	<i>L. acidophilus</i>	ATCC 4356	ATCC ^b
2	NRRL B-4435	<i>L. amylophilus</i>		Original isolate
3	NRRL B-4436	<i>L. amylophilus</i>		Original isolate
4	NRRL B-4437 ^a	<i>L. amylophilus</i>		Original isolate
5	NRRL B-4438	<i>L. amylophilus</i>		Original isolate
6	NRRL B-548	<i>L. bulgaricus</i>	ATCC 7943	ATCC
7	NRRL B-734	<i>L. bulgaricus</i>	ATCC 7994	ATCC
8	NRRL B-1909	<i>L. bulgaricus</i>	ATCC 12278	ATCC
9	NRRL B-1445	<i>L. casei</i>	ATCC 7496	Flynn
10	NRRL B-1913	<i>L. casei</i>	ATCC 11582	ATCC
11	NRRL B-1922 ^a	<i>L. casei</i>	ATCC 393	ATCC
12	NRRL B-4564 ^a	<i>L. casei</i> subsp. <i>alactosus</i>	ATCC 27216	ATCC
13	NRRL B-4560	<i>L. casei</i> subsp. <i>pseudopiantarum</i>	ATCC 25598	ATCC
14	NRRL B-1041	<i>L. casei</i> subsp. <i>rharnosus</i>	ATCC 9595	ATCC
15	NRRL B-1914	<i>L. casei</i> subsp. <i>rharnosus</i>	ATCC 12116	ATCC
16	NRRL B-4561	<i>L. casei</i> subsp. <i>tolerans</i>	ATCC 25599	ATCC
17	NRRL B-4391 ^a	<i>L. coryniformis</i> subsp. <i>coryniformis</i>	ATCC 25602	ATCC
18	NRRL B-4390 ^c	<i>L. coryniformis</i> subsp. <i>torquens</i>	ATCC 25600	ATCC
19	NRRL B-4562 ^a	<i>L. curvatus</i>	ATCC 25601	ATCC
20	NRRL B-763 ^a	<i>L. delbrueckii</i>	ATCC 9649	ATCC
21	NRRL B-1933	<i>L. delbrueckii</i>	ATCC 11978	ATCC
22	NRRL B-1934	<i>L. delbrueckii</i>	ATCC 11979	ATCC
23	NRRL B-1842	<i>L. helveticus</i>	ATCC 8018	ATCC
24	NRRL B-1937	<i>L. helveticus</i>	ATCC 11982	ATCC
25	NRRL B-4526 ^a	<i>L. helveticus</i>	ATCC 15009	ATCC
26	NRRL B-4559 ^c	<i>L. homohiochi</i>	ATCC 15434	ATCC
27	NRRL B-4550 ^a	<i>L. jensenii</i>	ATCC 25258	ATCC
28	NRRL B-4525 ^a	<i>L. lactis</i>	ATCC 12315	ATCC
29	NRRL B-4523 ^a	<i>L. leichmannii</i>	ATCC 4797	ATCC
30	NRRL B-4563 ^a	<i>L. mali</i>	ATCC 27053	ATCC
31	NRRL B-4496 ^a	<i>L. plantarum</i>	ATCC 14917	ATCC
32	NRRL B-1950 ^a	<i>L. salivarius</i> subsp. <i>salicinius</i>	ATCC 11742	ATCC
33	NRRL B-1949 ^a	<i>L. salivarius</i> subsp. <i>salivarius</i>	ATCC 11741	ATCC
34	NRRL B-4538	<i>L. amylovorus</i>		Original isolate
35	NRRL B-4539	<i>L. amylovorus</i>		Original isolate
36	NRRL B-4540 ^a	<i>L. amylovorus</i>		Original isolate
37	NRRL B-4542	<i>L. amylovorus</i>		Original isolate
38	NRRL B-4449 ^a	<i>L. xylosus</i>	ATCC 15577	ATCC
39	NRRL B-4565 ^a	' <i>L. yamanashiensis</i> ' ^d	ATCC 27304	ATCC

^a Type strain.^b American Type Culture Collection.^c Reference strain.^d Not on the Approved Lists of Bacterial Names (19).

itored in a UV-VIS thermal analyzer system (Gilford Instrument Laboratories, Inc.). A denaturation temperature of 90°C was obtained rapidly and held for 10 min after the hyperchromic shift at 260 nm. Subsequently, the temperature was lowered rapidly to 25°C below T_m ; the optical density at this point was taken to represent 0% renaturation (although some undoubtedly occurs). For these studies, the T_m values were determined in the presence of 3× SSC and 20% dimethyl sulfoxide. The absorbance change at 260 nm was monitored until the DNA was more than 50% reassociated. All determinations were repeated three times. Percent renaturation was calculated by the equation given by Seidler and Mandel (17).

Cell wall analysis. A purified cell wall preparation was made from cells in the mid-log phase. The procedure of Cummins and Johnson (3) was used to disrupt the cells and to purify the cell walls obtained.

To determine the sugar composition, 10 mg of the cell wall preparation was hydrolyzed by heating at 100°C for 2 h in 2 N H₂SO₄. When cooled, the acid was neutralized with Ba(OH)₂. After filtration to remove the barium sulfate precipitate, the supernatant was evaporated to dryness. The soluble material was taken up in 0.2 to 0.3 ml of distilled water. The hydrolysates (25 µl) were spotted on Whatman no. 1 paper and developed with a butanol-pyridine-water (60:40:30) solvent. The sugars were detected with aniline hydro-

gen phthalate or with alkaline silver nitrate (20).

For amino acid analysis, the cell wall was hydrolyzed by refluxing in 6 N HCl for 24 h. Amino acid analysis of the hydrolysates was carried out in a Glenco MM-100 amino acid analyzer.

RESULTS AND DISCUSSION

The cell walls of all four *L. amylophilus* strains contained the sugars galactose, glucose, glucosamine, mannose, and rhamnose, and the amino acids alanine, aspartic acid, glutamic acid, and lysine. Visual inspection of the paper chromatograms indicated that glucose and galactose were the major sugar components of the cell wall. The average relative molar concentrations of aspartic acid, glutamic acid, lysine, and alanine were 0.37, 0.63, 0.40, and 1.0, respectively.

The amino acid content of *L. amylophilus* cell wall was typical of that of most homofermentative *Lactobacillus* species. In the cell walls of these organisms, D-aspartic acid and lysine consistently occur as linked moieties in the peptido-

glycan (15, 16). Aspartic acid acts as the bridge between peptide subunits (16). The equimolar amounts of aspartic acid and lysine found in the cell wall of *L. amylophilus* is what would be expected if these amino acids were linked. Experiments to show linkage of aspartic acid and lysine in *L. amylophilus* cell wall were not done in this study. In several exceptions, viz., *L. plantarum*, *L. ruminis*, *L. vitulinis*, and "*L. yamanashiensis*" (this name was not on the Approved Lists of Bacterial Names [19], has not been validly published since 1 January 1980, and hence is without standing in nomenclature), the cell walls contain diaminopimelic acid, which directly cross-links the peptide subunits (2, 16, 18). No diaminopimelic acid was present in *L. amylophilus* cell walls. The sugar contents of *L. amylophilus* and *L. casei* walls are similar (15).

Spectrophotometric techniques for determining the degree of DNA-DNA hybridization were discussed both by Seidler and Mandel (17) and by De Ley et al. (4). The C_{0t} technique (21) is the

TABLE 2. DNA reassociation of *L. amylophilus* and species with similar characteristics

Strain (serial no.)	Similarity	% Reassociation ^a with DNA from <i>L. amylophilus</i>			
		Serial no. 2	Serial no. 3	Serial no. 4	Serial no. 5
9	Produce L-(+)-lactic acid, mol% G+C	13	10	8	10
10		10	15	12	11
11		15	11	12	16
12		15	14	10	11
13		33	22	12	20
14		10	15	11	12
15		12	8	14	18
16		21	14	15	18
32	Produce L-(+)-lactic acid	17	12	15	13
33		12	10	9	10
38		16	5	13	7
6	Sugar fermentation pattern	8	10	15	6
7		7	9	11	8
8		12	12	10	13
18		11	8	11	11
20		11	8	11	11
21		8	8	8	10
22		10	12	9	7
23		14	10	13	9
24		15	9	10	10
25		8	10	7	15
34	Hydrolyze starch	12	11	13	15
35		10	9	10	9
36		10	9	11	12
37		11	10	10	14
2	Strains		92	90	95
3		90		95	90
4		91	93		98
5		94	90	96	

^a Average of three determinations.

basis for Seidler and Mandel's procedure, whereas the initial rate of reassociation concept (7) is the foundation for the method of De Ley et al. The present study employed the method of Seidler and Mandel. Although the membrane filter (6) and hydroxyapatite hybridization procedures (1) may permit somewhat more detailed examination of the DNA relationship involved, the spectrophotometric procedure is a simple, rapid, and reproducible method for distinguishing between species and groups within species (11).

The extent of DNA-DNA interaction between *L. amylophilus* and species with similar characteristics is recorded in Table 2. Whenever possible, each species is represented by the type (or reference) strain and several other strains.

Although the four *L. amylophilus* strains showed some variation in their patterns of sugar fermentation (14), they were closely related genetically, as demonstrated by the high (90 to 98%) DNA reassociation values (Table 2). These strains, however, are not genetically related to *L. amylovorus* strains (NRRL B-4538, B-4539, B-4540, and B-4552), starch-hydrolyzing lactobacilli isolated from cattle waste-corn fermentations, as was indicated by the low DNA reassociation levels (10 to 15%; see Table 2). Furthermore, *L. amylophilus* was phenotypically distinguishable from *L. amylovorus* by differences in several characteristics. *L. amylophilus* produced L-(+)-lactic acid from glucose, had a guanine plus cytosine (G+C) content of 45.5 mol%, and grew at 15°C but not at 45°C (14). In contrast, *L. amylovorus* produces DL-lactic acid, has a G+C content of 40.4 mol%, and grows at 45°C but not at 15°C (12). The sugar fermentation patterns of these species also differed significantly (13).

L. amylophilus is phenotypically distinct from the other species of *Lactobacillus* (13). However, similarities of certain selected characteristics suggest that *L. amylophilus* could be related to one of several *Lactobacillus* species. For example, *L. amylophilus* shares with *L. casei* and its subspecies, both subspecies of *L. salivarius*, and *L. xylosus* the ability to produce mainly L-(+)-lactic acid from glucose (14, 15). Moreover, the G+C contents of *L. casei* DNA (46.4 mol%) and of *L. amylophilus* DNA (45.5 mol%) differ only slightly (14, 15). The low range of DNA reassociation levels (5 to 33%) shown in Table 2 indicates that *L. amylophilus* is not closely related genetically to any of the species or subspecies studied that produce L-(+)-lactic acid.

Similarities of their sugar fermentation patterns suggest a possible relationship of *L. amylophilus* and *L. bulgaricus*, *L. coryniformis* subsp. *torquens*, *L. delbrueckii*, and *L. helveticus*. Four of these species ferment fructose,

galactose, glucose, mannose, and maltose; the fifth, *L. bulgaricus*, attacks the hexoses but not maltose (14, 15). Differentiation from *L. amylophilus* is thus based on the fermentation of sucrose by *L. delbrueckii*, of mannitol and gluconate by *L. coryniformis* subsp. *torquens*, and of lactose by both *L. bulgaricus* and *L. helveticus* (14, 15). None of the four species ferments starch. Furthermore, *L. amylophilus* and *L. coryniformis* subsp. *torquens* have similar G+C contents of about 45 mol% (14, 15). In spite of these similarities in fermentation patterns, *L. amylophilus* and the four species are not closely related genetically, as indicated by the low yield (6 to 15%) of DNA heteroduplex formation (Table 2).

DNA reassociation determinations were also carried out to assess the relationship between *L. amylophilus* and type strains of other homofermentative *Lactobacillus* species. Reassociation values ranged from a low of 5% to a high of 18% for the interaction of DNA from *L. amylophilus* with that of "*L. yamanashiensis*" B-4565 (serial no. 39) and that of *L. curvatus* B-4562 (serial no. 19), respectively (Table 3). These values indicate lack of genetic relationship between *L. amylophilus* and lactobacilli with which it shares no obvious similarities.

Apart from the genotypic homogeneity of the strains assigned to the species *L. amylophilus*, the present study showed an absence of a close genetic relationship between *L. amylophilus* and the other lactobacilli studied. Thus, it is concluded that *L. amylophilus* is a genospecies clearly distinct from the other species. It is also evident that similar fermentation patterns and other physiological characteristics and similar G+C ratios are not necessarily indicators of genetic relationship.

TABLE 3. DNA reassociation between *L. amylophilus* and other homofermentative species

Strain (serial no.)	% Reassociation ^a with DNA from <i>L. amylophilus</i>			
	Serial no. 2	Serial no. 3	Serial no. 4	Serial no. 5
1	15	13	9	10
17	15	11	17	12
19	18	12	15	19
26	7	8	7	9
27	14	12	8	11
28	8	8	10	9
29	15	18	17	15
30	8	8	9	7
31	17	17	10	15
39	5	7	7	8

^a Average of three determinations.

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REPRINT REQUESTS

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