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Taxonomy of the *Lactobacillus acidophilus* Group

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A total of 89 strains designated *Lactobacillus acidophilus* were examined for physiological properties, type of lactic acid produced, cell wall sugar pattern, guanine plus cytosine content of deoxyribonucleic acid (DNA), and DNA homology values compared with selected reference strains. Immunological reactions among a group of the strains were determined by gel diffusion tests, using antiserum to purified lactic acid dehydrogenase (LDH) from a single strain (Sharpe strain A18). Antiserum to glyceraldehyde-3-phosphate dehydrogenase from strain ATCC 4356 was used in microcomplement fixation tests to determine relationships among some strains. DNA preparations from 78 of the 89 strains of *L. acidophilus* were distributed among six distinct homology groups, designated A1, A2, A3, A4, B1, and B2. The A group strains had 20 to 30% intergroup homology but very low homology to groups B1 and B2. Likewise, the strains in the two B groups had 20 to 30% intergroup homology but very low homology to the A group strains. Nine strains did not fall into any of the six homology groups. The guanine plus cytosine contents of the DNAs in strains comprising the six homology groups varied from 32 to 38 mol%. In the nine strains not falling into any of the homology groups, the guanine plus cytosine contents were 39 to 47 mol%. Homology group A1, which includes the neotype strain of *L. acidophilus* (ATCC 4356), is very homogeneous, with most strains showing 95% or more homology to the reference strain. This group corresponds to LDH serogroup III. Strains in the other homology groups showed 60 to 90% homology to their reference strains. Strains of LDH serogroup II were found in homology groups A2, A3, and A4, and those in LDH serogroup I were in homology groups B1 and B2. In general, the glyceraldehyde-3-phosphate dehydrogenase serology results correlated well with the LDH results. Other phenotypic test results were similar for all of the DNA homology groups. It is recommended that homology group A1 be designated *L. acidophilus* and that strain ATCC 4356 remain the neotype strain.

In 1900, Moro isolated from infant feces gram-positive, asporogenous rods, which he named *Bacillus acidophilus* (26). In 1929, this species was included by Holland in the genus *Lactobacillus* (16). The original strain of Moro was probably lost (13), and its description, according to present day standards, is very incomplete. Moreover, investigators tended to identify all new *Lactobacillus* isolates from mouths and from intestinal and vaginal floras as strains of *Lactobacillus acidophilus*. It was only in 1970 that Hansen and Mocquot adequately described this species and designated a neotype strain for it (ATCC 4356) according to the recommendations of the International Subcommittee on the Taxonomy of Lactobacilli and Closely Related Organisms (13, 14). The description of *L. acidophilus* by Hansen and Mocquot was derived from the important taxonomic work of Rogosa and Sharpe (29), who based their description of the species on their own observations and on those of Curran et al. (5), Tittsler et al. (35), and Rogosa et al. (30).

The guanine plus cytosine (G+C) content of the deoxyribonucleic acid (DNA) of several strains of *L. acidophilus* shows a rather good homogeneity, ranging from 34 to 38 mol% (11, 12). However, several recent taxonomic investigations have suggested that this species might be heterogeneous. Among strains whose characters were in accord with the species description by Hansen and Mocquot (14), biotypes or groups were described successively on the basis of colonial morphology and substrate fermentations (28), cell width (W. E. C. Moore, personal communication), serological properties (6), cell wall antigens (32, 33), and electrophoretic and antigenic characteristics of D- and L-lactic acid dehydrogenases (LDH) (7, 9).

Preliminary DNA homology results indicated

that a genetic diversity existed among strains identified as members of *L. acidophilus*. These differences appeared to correlate with some of the immunological results. Therefore, our study was expanded to include additional culture collection strains of *L. acidophilus*, more of the strains that had been investigated immunologically, and some recently isolated strains from the collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (VPI), Blacksburg. Phenotypic properties were determined for all of the strains, cell wall sugars were determined for many of them, and immunological relationships of LDH and glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) enzymes were determined for representative strains from each DNA homology group.

MATERIALS AND METHODS

Bacterial strains. The *Lactobacillus* strains employed in this study are listed in Table 1. Strains designated with a VPI number only were either sent to the Anaerobe Laboratory for identification or were isolated at this laboratory. This table contains the reference collection abbreviations and numbers for those strains received from national culture collections or just strain numbers for strains obtained from personal collections. There appears to have been some confusion with some of the strains. For example, VPI strains 11084, 11085, and 11086 have all been cross-referenced as NCTC 1950 in culture collection catalogs. Numbers 11084 and 11085 may well represent the same strain since both are in homology group A-1; however, strain 11086 belongs to homology group A-2. Several of the strains were originally obtained from the American Type Culture Collection (ATCC) (VPI strains 0324, 0325, 0326, 0328, 0331, 0333, 11085, 11091, and 11093) but are no longer listed in the catalog of the ATCC.

Physiological properties. The physiological properties of the organisms were determined by L. V. Holdeman and W. E. C. Moore (15), and the isomers of the lactic acid produced were determined by E. Cato by a previously described method (15). All of the media used in these tests contained 0.1% Tween 80.

DNA isolation, G+C ratios, and DNA homologies. Each organism was grown in 2.5 liters of medium. The medium contained mineral salts (15), 1% Trypticase, 0.5% yeast extract, 1% glucose, 0.01% heme, 0.05% Tween 80, and 0.05 M potassium phosphate buffer (pH 7.0) and was prepared as described previously (4). For labeling of DNA, the yeast extract concentration was lowered to 0.05%, and 0.5 mCi of either [^{3}H]-thymidine or [^{3}H]adenine (New England Nuclear Corp., Boston, Mass.) was added to 40 ml of the medium.

Harvested cells were suspended in sodium chloride-ethylenediaminetetraacetic acid buffer, pH 8.0 (23), and were physically disrupted by shaking with glass beads (diameter, 0.074 to 0.105 mm; type IV C; Cataphote Corp., Jackson, Miss.) in a Braun cell disintegrator. The cells were shaken for 5 min at 4,000 cycles per min. The disrupted cells were separated from the glass beads by filtration through a coarse scintered glass filter, and sodium dodecyl sulfate (final concentration, 1%) and 50 μg of pronase (Calbiochem, La Jolla, Calif.) per ml were added. After incubation at 56°C for 1 h, the cell walls were removed by centrifugation. The cell walls were saved for sugar analysis (see below), and the supernatant was extracted with phenol. From this point the hydroxylapatite procedure was used for DNA isolation, as previously described (20).

The G+C content of each DNA preparation was calculated from the thermal melting temperature. The thermal melting temperature values were determined by using a Gilford model 2400 spectrophotometer equipped with a model 2527 thermal programmer. Details of the procedure have been published previously (21, 24).

The unlabeled DNA preparations, which were dissolved in 0.02 M NaCl-10^{-3} M HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0), were further fragmented by sonic oscillation. Each preparation was sonicated for two 30-s periods by a Biosonik III (Bronwill Scientific Inc., Rochester, N.Y.) instrument fitted with a 0.375-inch (ca. 0.95-cm) diameter tip and at an energy setting of 60%. The DNA preparations were then denatured, and any contaminating ribonucleic acid was hydrolyzed by the addition of 5 N NaOH to a final concentration of 0.25 N. After heating for 15 min at 50°C, the preparations were cooled, and the NaOH was neutralized with 5 N HCl. The DNA preparations were then dialyzed overnight against 0.02 M NaCl-10^{-3} M HEPES buffer (pH 7.0) and then adjusted to 0.6 mg/ml. The labeled DNA preparations were prepared in the same manner, except that the DNA was fragmented by two passages through a French pressure cell at a pressure of 1.1 kbar and the final DNA concentration was adjusted to 0.01 mg/ml.

DNA homology experiments were performed by a variation of the S1 nuclease procedure as described by Crosa et al. (3). Free solution reassociation reactions were carried out by using vials (6 by 25 mm) which were capped with the insertion part of serum bottle stoppers. The reaction mixtures each contained 10 μl (0.1 μg) of labeled DNA, 50 μl (30 μg) of unlabeled DNA (either homologous, heterologous bacterial, or native fragmented salmon sperm DNA), and 50 μl of 0.88 M NaCl-10^{-3} M HEPES buffer (pH 7.0). Just before each experiment, the amount of labeled DNA needed for the experiment was denatured a second time by heating the tube in a boiling water bath for 2 to 3 min. The reaction vials containing native salmon sperm DNA were used to measure the amount of self renaturation of labeled DNA during the incubation period. The reaction vials were incubated for 16 h at a temperature 25°C below the thermal melting temperature of the reference DNA (as measured in 0.15 M NaCl-0.015 M sodium citrate buffer, pH 7.0). After incubation, a 100-μl sample was removed from each vial and transferred into a test tube (13 by 100 mm) for S1 nuclease digestion.

The S1 nuclease was either prepared from crude amylase powder (type IVA; Sigma Chemical Co., St. Louis, Mo.) by the method of Vogt (36) but with no further purification after elution from the diethyl-

TABLE 1. *List of strains used in this study*

VPI no.	Other no.	Source and other comments
Homology Group A-1		
6032	ATCC 4356	Human, neotype strain, strain scav, NCIB 8690
0331	ATCC 4796	E. B. Fred L.d.4
0330[a]	ATCC 4357	Human, Kulp strain PAK
0328	ATCC 4355	Rat, Kulp strain R-1-1
0326	ATCC 832	Rat, L. F. Rettger 4B, NCIB 1723
0324	ATCC 314	Human, E. O. Jordan, L. F. Rettger 43
11084[b]	NCTC 1899	Strain 2 Kopeloff, ATCC 11975, NCTC 1950, NCIB 1899
11085[b1]	ATCC 11975	NCIB 1899, NCTC 1899
11091	ATCC 9224	M. Rogosa *L. jugerti*
11096[a1]	NCIB 8607	Strain PAK, ATCC 4357
11473		Inoculum for sweet acidophilus milk
11566		Sweet acidophilus milk
11695	SA18	F. Gasser, M. E. Sharpe A18
11760[c]	NCDO 1	M. E. Sharpe A1
11843[c1]	A1	M. E. Sharpe
12596	YIT 0165	H. Shimohashi, fermented milk
Homology group A-2		
1784		Chicken cecum
1799		Chicken crop
11083	NCTC 2949	Cruickshank V.3, human vagina
0607-1B		Unknown
11086[b2]	NCIB 4505	(Strain 7, NCTC 4505, NCDO 5) NCTC 1950
11082	NCTC 4504	Cruickshank strain 2, NCIB 4504, NCDO 4
6272		Human, cervical vagina
11090	NCIB 8821	Strain Hayward 1F, human saliva
6317		Human, cervical vagina
7635		Human, urine
11697	SA 5	F. Gasser, M. E. Sharpe A5
11845	A 4	M. E. Sharpe
11761	SA 11	F. Gasser, M. E. Sharpe A11
Homology group A-3		
1754		Hog, small intestine
0818		Hog, small intestine
1756		Hog, small intestine
0824		Hog, small intestine
0773		Hog, small intestine
0673		Hog, small intestine
1830		Chicken crop
P9A-27		Hog, stomach
P10B-17		Hog, small intestine
Homology group A-4		
1294		Chicken crop
1793		Chicken large intestine
2164A		Turkey cecum (with enteritis)

TABLE 1. *Continued*

VPI no.	Other no.	Source and other comments
Homology group B-1		
6033	ATCC 19992	Feces, P. A. Hansen ISL 4, G. Reuter F164
0334	ATCC 4963	Human, L. F. Rettger 5
11092	NCIB 8820	Strain Hayward 1C, ATCC 29601, carious tooth
11087	NCIB 8819	Strain Hayward 1A, human saliva
0333	ATCC 4962	L. F. Rettger 3
11089	ATCC 9857	Döderlein's bacillus, NCTC 2948
6099B		Human, stomach
9395		Human, abdominal wound
7978C		Human, throat fistula
6325		Human, cervical vagina
6077		Human, stomach
6322		Human, cervical vagina
7763		Human, vaginal swab
6364A		Human, cervical vagina
6324		Human, cervical vagina
7227		Human, urine
0657	CDC 1191	Human, blood
7677		Human, gastrostomy
9940		D. W. Lambe, 1464-74B
8832		Human, pelvis pus
6101		Human, stomach, sprue
11757	65K	F. Gasser
11759[d]	NCDO3	M. E. Sharpe
11844[d1]	A3	M. E. Sharpe
12598	LA-2	H. Shimohashi, human feces
12599	LA-3	H. Shimohashi, human feces
12601	YIT-0164	H. Shimohashi, fermented milk
Homology group B-2		
7893A		Human, sinus drainage pus
7960		Human, blood
0779		Hog cecum
0325	ATCC 332	L. A. Rogers (USDA), L. F. Rettger
11088	ATCC 11506	NCIB 8795, NCIB 8892
11694	SA20	F. Gasser
11696	63E	F. Gasser
11846	A21	M. E. Sharpe
12597	RA-69	H. Shimohashi, rat intestinal tract
12600	RA-3	H. Shimohashi, rat intestinal tract
12602	RA-42	H. Shimohashi, rat intestinal tract
12603	RA-22	H. Shimohashi, rat intestinal tract
Other *L. acidophilus* like organisms		
0776		Hog cecum
1309		Chicken, small intestine
1395		Dog, small intestine
1796		Chicken crop
8409		Mouse cecum
9412		Bovine rumen
11093	ATCC 4913	E. McCoy L.d.3, *L. acidophilus*, amino acid assay
11094	NCTC 1406	Type 1, *Lactobacillus acidophilus-odontolyticus*
11095	NCTC 1407	Type 2, *Lactobacillus acidophilus-odontolyticus*

TABLE 1. *Continued*

VPI no.	Other no.	Source and other comments
Other species		
6044	ATCC 15009	Neotype strain *Lactobacillus helveticus*
11343	ATCC 25258	Type strain *Lactobacillus jensenii*

[a] Numbers with superscripts *a* and *a1* represent a single strain.
[b] Numbers with superscripts *b*, *b1*, and *b2* represent a single strain.
[c] Numbers with superscripts *c* and *c1* represent a single strain.
[d] Numbers with superscripts *d* and *d1* represent a single strain.

aminoethyl column or purchased from Calbiochem. In either case, the nuclease activities were titrated by using 50 μl of twofold dilutions of the enzyme preparations to degrade 30 μg of denatured DNA in 1.0-ml volumes of 0.05 M sodium acetate–0.3 M sodium chloride–0.5 mM zinc chloride buffer, pH 4.6 (31). Digestion was for 1 h at 50°C. All assays were then performed by using the enzyme at twice the concentration required for effective hydrolysis of 30 μg of single-stranded DNA.

In addition to the sample of reassociation mixture, each digestion tube contained 1.0 ml of the sodium acetate-sodium chloride-zinc chloride buffer, 25 μg of fragmented, denatured salmon sperm DNA, and 50 μl of S1 nuclease. After incubation for 1 h at 50°C, an equal volume of 10% trichloroacetic acid was added to each tube. The tubes were cooled in a refrigerator at 4°C for at least 1 h, and the precipitates were collected on nitrocellulose membrane filters (BA85; Schleicher & Schuell Co., Keene, N.H.). The membranes were dried, and the radioactivity was measured with a liquid scintillation counter and a toluene-based scintillation fluid.

Cell wall analysis. The methods for the preparation of purified cell walls and determination of cell wall sugars have been described previously (4).

Immunological procedures. (i) GA3PDH. An electrophoretically homogeneous preparation of GA3PDH was prepared from *L. acidophilus* ATCC 4356 by an adaptation of a procedure used to purify the same enzyme from *Streptococcus faecalis* (N. M. Chace and J. London, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K48, p. 155). High-titer antiserum was produced in a pair of 6-month-old Australian white rabbits by a series of four weekly intradermal injections of a suspension containing 0.178 mg of GA3PDH, 20 μg of methylated bovine serum albumin, and 0.2 ml of complete Freund adjuvant in a final volume of 0.6 ml. The intradermal injections were followed by three intravenous injections given 1 week apart and consisting of 0.148 mg of enzyme plus 20 μg of methylated bovine serum albumin. At 5 days after the final injection, the rabbits were bled from the central ear artery, and 40 ml of blood was collected. After standing overnight at 4°C, the clotted erythrocytes and fibrin were separated from the plasma by centrifugation, and the clarified serum was stored as 5-ml volumes at −40°C.

Immunodiffusion experiments were carried out according to the Stollar and Levine (34) modification of the Ouchterlony technique. Details of these experiments are presented elsewhere (22). The convention of Gasser and Gasser (9) was used to summarize immunodiffusion results. Interpretation of these results has also been described previously (22).

Microcomplement fixation experiments were performed by the procedure of Wasserman and Levine (37); minor modifications of this technique have been described elsewhere (22). The GA3PDH antiserum was used at a dilution of 1:120,000 to 1:150,000 with the homologous antigen; the latter was employed in the range of 1 to 10 μg of protein. Heterologous antigen was used in the range of 0.5 to 50 μg. The relative amounts of complement fixed by both homologous and heterologous antigens were linear and paralleled functions of the logarithm of the antibody concentration. Therefore, results of the experiments with different antigens were directly comparable (31). The index of dissimilarity for each heterologous aldolase was calculated from those data by the equation of Champion et al. (2). For the anti-*L. acidophilus* GA3PDH, the term *m* of their equation equaled 247. Data are also expressed as immunological distance units; these are derived by multiplying the log of the index of dissimilarity by 100 (2).

(ii) LDH. The presence of L-LDH and D-LDH (both nicotinamide adenine dinucleotide-dependent and -independent) activity in crude extracts was determined spectophotometrically (7). Immunological groups were determined with immunodiffusion experiments by using antiserum produced against the L-LDH of *L. acidophilus* A18 (=VPI 11695) according to a technique described previously (8, 9). Each unknown extract was placed into a well next to another reference crude extract. The reference strains and the immunological groups are as follows: group I, 65K (=VPI 11757) and 63E (=VPI 11696); group II, A5 (=VPI 11697) and A11 (=VPI 11761); and group III, A18 (=VPI 11695) and ATCC 4356 (=VPI 6032). Moreover, every unknown crude extract was tested by pairwise comparison against all of the other unknown extracts in order to confirm clearly its appurtenance to group I, II, or III.

RESULTS

The G+C values and the DNA homology results for the *L. acidophilus* group are presented in Table 2. The G+C values are in good agreement with those obtained by Gasser and Mandel (11) and by Gasser and Sebald (12) in those cases where the same organisms were examined.

TABLE 2. *DNA homologies among the L. acidophilus group of organisms*

VPI no.	Other designations	GC (mol %)	Percent homology to: 6032	1784	7635	1754	1294	6033	6322	7960
Homology Group A-1										
6032	ATCC 4356	32	100	35	26	25	19	10	10	6
0331	ATCC 4796	33	100	27	25	22	21	6	10	5
0330	ATCC 4357	32	96	27	23	24	20	11	14	6
0328	ATCC 4355	33	94	28	36	23	20	6	11	2
0326	ATCC 832	33	96	28	31	34	22	8	12	2
0324	ATCC 314	31	104	15	21	26	18	6	6	6
11084	NCTC 1899	35	99	17	20	27	23	5	7	5
11085	ATCC 11975	36	102	2	20	28	25	5	4	4
11091	ATCC 9224	36	98	20	23	28	25	8	6	0
11096	NCIB 8607	36	97	20	22	30	23	5	4	-2
11473		34	92	24	22	34	24	6	6	11
11566		34	102	26	23	30	24	9	9	15
11695	SA18	37	87	18	21	35	20	0	8	1
11760	NCDO1	34	93	7	22	34	23	13	10	15
11843	A1	33	97	18	25	32	29	7	5	3
12596	YIT 0165	34	95	14	19	24	21	5	6	10
Homology Group A-2										
1784		35	29	100	71	22	18	8	10	0
1799		36	23	93	76	27	24	9	10	2
11083	NCTC 2949	38	26	69	70	23	24	4	5	7
0607-1B	CDC	35	23	65	60	21	21	8	9	5
11082	NCTC 4504	38	22	62	66	20	24	9	8	0
11086	NCIB 4505	37	23	62	70	22	24	3	7	0
6272		35	21	60	78	20	20	10	8	2
11090	NCIB 8821	38	22	59	72	24	21	9	8	4
6317		37	20	57	69	19	17	6	7	0
7635		36	21	57	100	18	24	5	8	6
11697	SA5	36	26	43	72	28	26	10	8	10
11845	A4	35	14	66	71	28	23	6	9	3
11761	SA11		23	63	71	28	16	1	8	4
Homology Group A-3										
1754		38	29	16	31	100	27	8	10	9
0818		36	27	17	26	88	29	7	8	7
1756		34	31	32	36	86	31	5	10	4
0824		36	27	30	33	84	30	4	9	2
0773			26	12	20	84	25	6	3	3
0673		38	21	17	20	80	25	6	4	4
1830			26	14	27	64	33	6	8	8
P9A-27		37	25	17	24	72	32	7	7	8
P10B-17		38	28	21	25	81	32	6	6	11
Homology Group A-4										
1294			27	13	22	30	100	5	5	7
1793			23	16	26	29	81	5	5	7
2164A			26	31	23	26	72	5	12	4
Homology Group B-1										
6033	ATCC 19992	33	9	11	14	6	6	100	64	32
0334	ATCC 4963	34	13	12	21	7	8	88	65	34
11092	NCIB 8820	35	11	9	9	9	8	85	56	32
11087	NCIB 8819	35	10	6	10	7	9	83	57	32
0333	ATCC 4962	33	11	12	20	9	7	80	68	33
11089	ATCC 9857	35	11	12	10	9	8	79	55	20
6099B		33	16	9	18	2	8	75	72	32
9395		33	7	2	16	2	8	87	57	35
7978C		33	6	4	12	6	7	77	55	33
6325		34	14	4	15	3	12	71	57	40
6077		34	17	10	11	4	10	70	64	31
6322		33	15	13	28	9	11	63	100	39
7763		32	9	9	16	4	7	58	92	33
6364		33	9	1	14	5	10	56	92	32
6324		33	8	2	14	4	10	57	91	36
7227		33	9	1	19	4	11	59	88	37
0657		33	7	5	10	0	6	64	81	35

TABLE 2. *Continued*

VPI no.	Other designations	GC (mol%)	Percent homology to: 6032	1784	7635	1754	1294	6033	6322	7960
7677			16	9	24	5	6	60	76	31
12598	LA-2	35	4	6	8	12	8	87	53	33
12601	YIT-0164	35	8	4	8	9	8	73	60	39
9940		32	10	11	19	11	12	68	66	36
8832		34	7	4	16	1	8	58	67	35
6101		34	8	2	19	4	10	53	68	36
11757	65K		9	17	11	12	9	77	62	44
11759	NCDO3		11	1	11	10	11	70	52	45
11844	A3	34	0	3	10	8	6	81	57	34
12599	LA-3	33	0	3	8	1	8	61	81	34
Homology Group B-2										
7893A		33	7	1	11	1	5	32	24	98
7960		33	8	2	18	0	7	32	27	100
0779		32	7	4	6	3	8	28	24	78
0325	ATCC 332	33	11	9	14	6	5	29	36	75
11088	ATCC 11506	32	6	10	5	4	5	18	22	67
11694	SA 20	30	15	8	11	11	9	36	30	79
11696	63 E	36	15	1	11	15	8	33	30	88
11846	A21		10	4	8	10	5	25	34	73
12597	RA-69	32	10	4	8	9	8	39	29	72
12600	RA-3	33	6	6	9	5	7	33	27	70
12602	RA-42	33	10	3	10	8	9	29	25	61
12603	RA-22	34	0	5	10	3	11	35	29	70
Other										
0776		47	0	0	10	1	4	4	3	0
1309		40	3	5	16	2	7	13	14	29
1395		41	13	1	6	0	3	0	5	0
1796		43	4	1	6	1	5	1	3	1
8409		39	1	0	4	0	3	0	2	0
9412			3	0	8	3	5	0	2	0
11093	ATCC 4913	49	0	3	5	6	5	1	0	0
11094	NCTC 1406	44	2	3	4	4	6	0	0	0
11095	NCTC 1407	46	1	3	5	2	3	3	0	0
6044	ATCC 15009	37	13	25	21	18	44	6	8	6
11343	ATCC 25258	31	11	4	7	7	7	8	6	7

The range of G+C values for a given DNA homology group appears to be greater than for other microorganisms that we have investigated. These variations may have been due to subtle differences in the DNA preparations, such as varying amounts of contaminating polysaccharide or teichoic acid or endogenous deoxyribonuclease activity, which may have slightly influenced some of the thermal melting temperature values. In homology group A-1, for example, DNA preparations ranging from 31 to 36 mol% G+C all had essentially 100% homology to reference strain 6032, so there is no reason to believe that there are real differences in the G+C values among strains within a DNA homology group.

DNA preparations from the *L. acidophilus* group are distributed among six distinct homology groups (Table 2). The first four, designated A-1, A-2, A-3, and A-4, had 20 to 30% intergroup homology but very low homology to the other two groups, B-1 and B-2. Likewise, DNA preparations from organisms in the B-1 and B-2 homology groups had 20 to 30% DNA homology to each other and very low homology to the four A groups.

Nine strains phenotypically similar to *L. acidophilus* were also included in the homology study. DNA from most of these organisms had higher G+C values than did DNA from the *L. acidophilus* group, and the DNA preparations had little or no homology to any of the reference DNA preparations.

The physiological properties of the 89 strains used in this study are presented in Table 3. All of the strains produced major amounts of lactic acid, moderate amounts of acetic acid, no hydrogen, and no catalase. Growth of all of the strains was enhanced by a fermentable carbohydrate, and all fermented cellobiose, fructose, glucose, and maltose and hydrolyzed esculin. None of the strains fermented inositol, produced indole, H_2S, lipase, lecithinase, or urease, or digested gelatin, milk, or meat. None of the strains attacked threonine or arabinose galactan. Two of the nine phenotypically similar strains were motile. All

TABLE 3. *Physiological characteristics of L. acidophilus homology groups*

Characteristic[d]	A-1(16)[a]	A-2(13)	A-3(9)	A-4(3)	B-1(27)	B-2(12)	Others(9)
Formic acid detected	3/16[b]	0/13	1/9	1/3	1/27	1/12	0/9
Succinic acid detected	13/16	13/13	9/9	3/3	23/27	10/12	5/9
Amygdalin	15/16	7/13	7/9	1/3	27/27	8/12	4/9
Arabinose	1/16	0/13	0/9	1/3	0/27	0/12	2/9
Erythritol	0/16	2/13	0/9	0/3	2/27	1/12	0/9
Esculin	13/16	13/13	7/9	3/3	25/27	11/12	7/9
Glycogen	16/16	8/16	2/9	2/3	2/27	1/12	0/8
Lactose	15/16	13/13	9/9	2/3	27/27	12/12	8/9
Mannitol	0/16	0/13	1/9	0/3	0/27	1/12	3/9
Mannose	16/16	13/13	9/9	3/3	26/27	12/12	9/9
Melezitose	1/16	1/13	0/9	0/3	3/27	2/12	1/9
Melibiose	9/16	9/13	6/9	1/3	2/27	6/12	8/9
Pectin	2/14	2/12	0/7	0/3	2/24	2/10	1/9
Raffinose	11/16	11/13	7/9	3/3	1/27	6/12	7/9
Rhamnose	1/16	0/13	0/9	0/3	0/27	0/12	1/9
Ribose	2/16	1/13	0/9	0/3	1/27	0/12	6/9
Salicin	15/16	13/13	9/9	3/3	27/27	12/12	9/9
Sorbitol	0/16	0/13	0/9	0/3	2/27	0/12	0/9
Starch	13/16	13/13	9/9	3/3	27/27	11/12	2/9
Starch hydrol.	4/16	10/13	9/9	3/3	12/27	0/12	7/9
Sucrose	16/16	13/13	9/9	3/3	27/27	12/12	7/9
Trehalose	14/16	9/13	1/9	0/3	27/27	10/12	6/9
Xylan	1/14	9/13	2/7	1/2	1/24	0/7	0/8
Xylose	1/16	2/13	0/9	0/3	4/27	3/12	0/9
Milk curd	15/16	13/13	9/9	1/3	25/27	12/12	7/9
NO_3 reduced	1/16	0/11	0/9	0/3	0/27	0/12	2/9
Good growth in bile	4/16	6/10	5/7	2/3	9/21	6/12	4/9
Hemolysis (alpha or atipical B)	3/9	6/11	6/7	2/3	19/26	6/12	5/9
Gas (trace - moderate)	5/15	8/12	7/9	2/3	18/27	7/12	7/9
NH_3 from arginine	2/15	0/12	1/9	0/3	1/27	0/12	0/9
Clindamycin resistant[c]	6/14	0/11	0/7	0/2	14/26	0/12	0/8
Penicillin G resistant[c]	0/14	4/11	5/7	2/2	1/26	0/12	2/8
Tetracycline resistant[c]	2/14	4/11	7/7	2/2	2/26	4/12	4/8
Pyruvic attacked	0/9	9/10	2/7	0/3	6/22	2/7	8/9
Grow at 45°C	10/12	13/13	9/9	2/2	25/25	7/8	7/9
Grow aerobically	9/16	9/13	6/9	3/3	18/27	12/12	6/9
Grow in candle jar	13/16	13/13	9/9	3/3	26/27	12/12	9/9

[a] Total numbers of strains tested are in parentheses.
[b] Number of strains positive/number of strains tested.
[c] Broth-disk method (15). Growth in medium containing clindamycin, 1.6 μg/ml; penicillin G, 2 units/ml; and tetracycline, 6 μg/ml.
[d] Acid production from substance specified, unless otherwise stated.

of the strains were susceptible to 12 μg of chloramphenicol per ml and 3 μg of erythromycin per ml (Table 3).

The lactic acid produced by all of the strains examined was a racemic mixture. The proportions of L to D optical isomers are presented in Table 4. The values for individual strains range from predominantly L+ isomer to predominantly D−, but the majority of the strains produced nearly equal amounts of the two isomers

TABLE 4. *Optical isomers of lactic acid produced by the homology groups of L. acidophilus*

Homology group	Amount of acid that is L isomer: (75+%)	(60-75%)	(40-60%)	(40-25%)	(30-0%)	Total number of strains
A-1	1[a]	2	4	8	1	16
A-2			5	8		13
A-3			2	6	1	9
A-4		2	1			3
B-1			7	18	2	27
B-2			5	6	1	12
Other		3	3	2	1	9

[a] Number of strains.

or a slight excess of the D− isomer.

The cell walls from this group of organisms invariably contain glucose, usually contain galactose, and occasionally contain mannose and/or rhamnose (Table 5). In general, walls from organisms in homology groups A-1 and A-2 contain less glucose than do walls from organisms in the other homology groups, although the amount varied from a trace to 3+ in these groups. It may be noted also that rhamnose was not found in the cell walls of any member of homology groups A-1 and A-2. In the other groups, rhamnose was present in some strains but not in others; however, it was not possible to correlate its presence clearly with any other characteristic of the strains. None of the strains examined contained diaminopimelic acid in its cell wall; however, the detailed structure of the peptidoglycan was not studied.

GA3PDH. Immunodiffusion experiments with a series of multiple cross-matches were carried out by using cell-free extracts of strains from the six DNA homology groups of *L. acidophilus*. Figure 1 shows the antigenic hierarchy of the various strains and reveals the following order: ATCC 4356 (=VPI 6032) > 1754 > 1294 > 6033 = or < 7960 > 1784.

Results obtained from microcomplement fixation experiments agree well with data from immunodiffusion experiments. Table 6 summarizes both the indexes of dissimilarity and immunological distances among the organisms. There appears to be little, if any, antigenic difference between strains 6033 and 7960 when strain 6032 is used as a point of reference. However, significant antigenic differences may actually exist. The degree of heterogeneity within *L. acidophilus* can be more fully appreciated by comparing the immunological distances presented in Table 6 with values obtained for *Lactobacillus helveticus* ATCC 15009 and *Lactobacillus jensenii* ATCC 25258 (unpublished data). These two species gave immunological distance values of 33 and 13.9, respectively.

LDH. All of the strains tested exhibited D-LDH activity, and all except 9940, 0328, and 11473 exhibited nicotinamide adenine dinucleotide-dependent L-LDH activity. A summary of the immunological results with L-LDH antiserum and of the relationship between immunological groups and DNA homology groups is shown in Table 7. The antiserum used was prepared against dehydrogenase from Sharpe strain A18 (=VPI 11695) in homology group A-1. Except for strain 11086, there is excellent correlation between the immunological groups based on LDH and those recognized by homology tests, although there is not necessarily a one-to-one relationship between the two groupings. Strains in LDH immunological group I are all in homology groups B-1 and B-2; strains in LDH immunological group II (except 11086) are in homology groups A-2, A-3, and A-4; and LDH immunological group III strains are all in homology group A-1.

DISCUSSION

The S1 nuclease procedure was used to determine DNA homology values in this study. For homology values up to about 60 or 70%, results with this method are about 20% lower than those obtained by either the hydroxylapatite or the membrane competition procedures (unpub-

TABLE 5. *Cell wall sugars in L. acidophilus strains*

Homology group	VPI no.	Other designations	Sugars in cell wall hydrolysates			
			Galactose	Glucose	Mannose	Rhamnose
A-1	6032	ATCC 4356	tr	+		
	0331	ATCC 4796	+	++	+	
	0330	ATCC 4357	tr	±		
	0328	ATCC 4355		±		
	0326	ATCC 832	±	+		
	0324	ATCC 314	±	+		
	11084	NCTC 1899	±	±		
	11085	ATCC 11975	±	±		
	11091	ATCC 9224	±	±		
	11096	NCIB 8607	±	±		
	11473			+		
	11566			+		
	11695	SA18	Tr	±		
	11760	NCDO1	±	++		
	11843	A1				
	12596	YIT 0165	+	+		
A-2	1784		+	+		
	1799		±	±		
	11083	NCTC 2949		±		
	0607-1B		±	±		
	11082	NCTC 4504	+	+		
	11086	NCIB 4505	±	±		
	6272			±		
	11090	NCIB 8821	±	±		
	6317			±	Tr	
	7635		±	±		
	11697	SA5	+	+		
	11845	A4				
	11761	SA11				
A-3	1754		+++	+++		+
	0818		±	+	±	
	0824		±	+++		+
	0773		±	++		
	0673			+		
	1830			+	Tr	
A-4	1294		+	+		
	1793		++	++		
	2164A		+	±		±
B-1	6033	ATCC 19992	Tr	+++		
	11092	NCIB 8820	+	++		
	11087	NCIB 8819	+	++		+
	0333	ATCC 4962	±	++		+
	11089	ATCC 9857	Tr	++		
	6099B		+	+++		
	9395			++		
	7978C		+	++		
	6325			++		
	6077		+	++		
	6322			+++		

TABLE 5. *Continued*

Homology group	VPI no	Other designations	Sugars in cell-wall hydrolysates			
			Galactose	Glucose	Mannose	Rhamnose
	7763			+		
	6364			++		
	6324			++		
	7227			+		
	0657			++		
	7677			+		
	12598	LA-2	+	++		
	12599	LA-3	+	++		
	12601	YIT-0164		++		
	9940			++		
	8832		+	+++		+
	6101		±	++		
	11757	65K	+++	+++		
	11759	NCDO3	Tr	+++		
	11844	A3				
B-2	7893A		±	++		
	7960		++	+++		
	0779		±	+		
	0325	ATCC 332	+	+		+
	11088	ATCC 11506	++	+++		+
	11694	SA 20	++	++		
	11696	63 E	+++	+++		
	11846	A21				
	0776		±	±		
	1309		+	+++		
	1395		+	++		
	1796		+	++		
	8409		+	+		+
	9412		Tr	+++		+
	11093	ATCC 4913	±	+		
	11094	NCTC 1406		+		
	11095	NCTC 1407		+		
	12597	RA-69	++	+		
	12600	RA-3	+	++		+
	12062	RA-42	++	++		++
	12603	RA-22	+	++		

lished data). Differences in the methods are less pronounced with higher (80 to 100%) homology values. When a comparison is made of the homology results from the *L. acidophilus* group with the homology results from other groups of bacteria (1, 3, 19, 20, 21, 27), each of the six *L. acidophilus* DNA homology groups could justifiably be designated as a separate species. Homology group A-1, which includes the neotype strain of *L. acidophilus* (ATCC 4356) as the reference strain, is very homogeneous. Most of the strains have greater than 95% DNA homology to the reference strain. This degree of homogeneity appears to be reflected in the similarity of their cell wall antigens (see below) and would explain why this group is easily identified by agglutination and precipitation tests (6, 33) (Table 8). Strains in the other DNA homology groups of *L. acidophilus* have from about 60% to 90% homology with their reference strains. This heterogeneity is within the range that has been found for most groups of bacteria considered to be single species.

Relationships between DNA homology groups and groups based on physiological tests. Reuter (28) grouped *L. acidophilus* strains into five biotypes, based primarily on variations in the fermentation of trehalose, mel-

ibiose, and raffinose. Mitsuoka (25) expanded the number of biotypes to 10 by also including variations in the coagulation of litmus milk (fermentation of lactose) and the fermentation of ribose. Eight of the strains used by Reuter and Mitsuoka were included in the present study. The results of physiological tests did not enable us to differentiate among the six DNA homology groups, since the degree of phenotypic heterogeneity within DNA homology groups was essentially the same as that observed among DNA homology groups. We must conclude, therefore, that the use of physiological tests, such as fermentation reactions and growth characteristics, are at present of little value in differentiating the various groups of *L. acidophilus*.

Relationship between homology groups and groups based on cell wall antigens. There have been several attempts to subdivide *L. acidophilus* by using serological methods; these include agglutination tests by Efthymiou and Hansen, (6) and immunodiffusion tests by Sharpe (32) and Shimohashi and Mutai (33). Some of the strains used in the studies of these authors were also used in the present investigation. The serological results are compared with the homology groups in Table 8. This table also includes the groups based on electrophoretic patterns of LDHs (7) wherever they have also been determined for the strains.

Sharpe (32 and personal communication) examined a group of 35 strains of *L. acidophilus*,

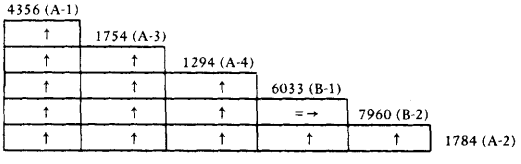

FIG. 1. *Hierarchical arrangement of L. acidophilus strains. Arrows indicate dominant antigen in cross-match.*

using HCl extracts of whole organisms. She found two groups which cross-reacted but which could be separated by the use of absorbed sera. However, almost one-half of the strains belonged to neither of these two groups and could not be classified. Sharpe designated the two cross-reacting groups I and II and the unclassified organisms as group III. Group I (10 of 35 strains) corresponds to homology group A-1 (group III of Gasser), and group II of Sharpe seems to correspond to homology group A-2. The two strains in group III of Sharpe (unclassified strains) which were examined in the present investigation both fall into homology group B-2.

Shimohashi and Mutai (33), using antigens extracted by hot dilute HCl and cold trichloroacetic acid, have also examined a series of *L. acidophilus* strains. Extracts were made from both cell walls and whole cells, and a number of distinct antigens (designated 3, 11, 12, 13, and 14) were recognized. Antigens 11, 12, 13, and 14 were found in both cell wall and whole cell extracts and are therefore presumably cell wall constituents. These four antigens were confined to strains of *L. acidophilus*. Antigen 3, on the other hand, and another antigen (not named) which was not found in cell walls appear to be rather widely distributed among lactobacilli; they are found not only in *L. acidophilus* but also in strains of *L. casei*, *L. bulgaricus*, *L. fermentum*, *L. salivarius*, *L. helveticus*, and *L. plantarum*.

Eight of the strains of Shimohashi and Mutai were examined in the present investigation, and there is good correlation between the homology groups and the distribution of cell wall antigens, especially antigens 11 and 12 (Table 8). It seems that antigen 12 is characteristic of group A strains and antigen 11 is characteristic of group B strains. Antigens 13 and 14 have so far been found in only a very limited number of strains.

Efthymiou and Hansen (6) examined the ag-

TABLE 6. *Immunological distances of the L. acidophilus GA3PDHs*

Strain	Index of dissimilarity	Immunologic distance
A-1 4356 (6032)	1.0	0
A-3 1754	1.26 + 0.08	10.08
A-4 1294	1.44 + 0.15	15.8
B-1 6033	1.99 + 0.08	29.8
B-2 7960	2.02 + 0.4	30.5
A-2 1784	3.45 + 0.6	53.8
ATCC 15009		33.0
ATCC 25258		13.9

TABLE 7. *L-LDH immunological groups of the L. acidophilus strains*

VPI strain no.	Immunologic group
Homology group A-1	
6031	III
0331	III
0330	III
0328	III
0326	III
0324	III
11084	III
11473	III
11695	III
Homology group A-2	
1784	II
11086	I
6317	II
7635	II
Homology group A-3	
1754	II
0824	II
Homology group A-4	
1294	II
2164A	II
Homology group B-1	
6033	I
6099B	I
6322	I
9940	I
8832	I
6101	I
11757	I
Homology group B-2	
7960	I
0325	I
11696	I

glutination reactions of a group of culture collection strains of *L. acidophilus*. They showed that ATCC strains 314, 832, 4356, and 4357 fell into one group serologically; in the present investigation all of these strains were found in homology group A-1. Strain ATCC 4962, which also belonged to this serological group, fell into group B-1 by homology, and it seems possible that the subculture of this strain which we examined was mislabeled. Efthymiou and Hansen also examined two other homology group B strains, ATCC 11506 (homology group B-2) and ATCC 4963 (homology group B-1), and found that these showed no cross-reaction with group A-1 strains. These agglutination results were not included in Table 8 because of the small number of strains involved, but they provide further evidence of the separate identity of homology group A-1 and distinguish it from groups B-1 and B-2.

Relationships between DNA homology groups and groups based on serology of LDH and GA3PDH. The precipitin reactions of the L-LDHs correlated in part with the DNA homology results. Of 27 strains, 26 could be correctly identified as belonging either to DNA homology group A-1, to homology group A-2, A-3, or A-4, or to homology group B-1 or B-2. These results are somewhat less specific than those obtained by Gasser and Hontebeyrie (10, 18) in the genus *Leuconostoc*. They found complete correlation between four serological groups and four DNA homology groups, with intergroup homology values similar to those found in the present study. There appears to be a common set of determinants in the A-2, A-3, and A-4 homology groups that react with the antibody produced against the A-1 strain (strain A18 of Sharpe). Similarly, there appears to be a set of antigenic determinants common to strains from the B-1 and B-2 homology groups which react with the antiserum. We might predict that the use of an antiserum produced against L-LDH from a strain in the A-2 homology group would permit accurate identification of strains in that group, whereas strains in homology groups A-1, A-3, and A-4 would be indistinguishable from each other. One might also predict that the use of antisera to L-LDH from a B-1 strain would distinguish the two B homology groups but would react equally with all A homology groups.

The immunological results with antiserum to GA3PDH from strain VPI 6032 were similar in most cases to those obtained when L-LDH antiserum was used. For example, the immunological distance of the two B strains (6033 [B-1] and 7960 [B-2]) from strain 6032 is considerably greater than the distance of the two A strains (1754 [A-3] and 1294 [A-4]) from 6032. The notable exception was for strain 1784, which belongs to homology group A-2 and to L-LDH immunological group II. The GA3PDH from this strain appears to be more distantly related to the reference enzyme (strain 6032, homology group A-1) than to the enzymes from organisms in homology groups B-1 and B-2 and from the type strains of *L. helveticus* (ATCC 15009) and *Lactobacillus jensenii* (ATCC 25258). It is possible that this discrepancy is due to a technical error, but if not, it will be of interest to determine whether it is characteristic of the A-2 homology group in general. The rest of the GA3PDH results correlate with the L-LDH results. This is similar to the complete correlation that was found when D-LDH and glucose-L-phosphate

TABLE 8. *Relationships between homology groups, serologic groups, and electrophoretic pattern of LDH*

Strains	Homology group	Antigen no. (Shimohashi & Mutai)	Electrophoretic pattern of LDH (Gasser)	Antigen group (Sharpe)
ATCC 4356		12	III	I
ATCC 4357		12		
Yito 165	A1	12		
A1		12	III	I
A18			III	I
A4		13	II	II
A5	A2		II	II
A11		12	II	II
ATCC 4963		11		
ATCC 9857		11		
ATCC 19992		11	Ib	
Yito 164	B1	11		
LA-2		11		
LA-3		11		
NCDO 3			Ia	
65K			Ia	
ATCC 11506		11		
RA-22		11		
RA-3		11		
RA-42		11		
RA-69	B2	14		
63-E			Ib	
A-20			Ib	III
A-21			Ib	III

dehydrogenase were used in the genus *Leuconostoc* (17).

DNA from *L. helveticus* strain ATCC 15004 has from 13 to 44% homology to the reference strains of the A homology groups and could be considered another A homology group. It is not surprising that the GA3PDH enzymes have immunological determinants in common. DNA homology between the various *L. acidophilus* reference strains and an *L. jensenii* strain (type strain; ATCC 25258) was hardly detectable, yet the immunological distance between it and *L. acidophilus* strain ATCC 4356 was only 13.9. It appears that the DNA coding for this enzyme is more conserved between these two organisms than is the bulk of the DNA.

Taxonomic recommendations. At the present time we have a number of taxonomic recommendations to make. First, DNA homology group A-1 (immunological group III of Gasser) should be designated *L. acidophilus* and strain ATCC 4356 should remain the neotype strain. This is in agreement with the proposal of Hansen and Mocquot (14). Second, DNA homology group A-2 should be considered a species (unnamed at this time) and strain ATCC 33197 (=VPI 7635) should be designated the type strain. This species would be in immunological group II of Gasser. Third, we recommend that DNA homology groups A-3 and A-4 should not be proposed as separate species until more strains have been investigated. This would allow time for an assessment of their ecological importance. Representatives of these groups are also being deposited with the ATCC. Fourth, we recommend that DNA homology group B-1 be designated a new species (also unnamed at this time). Kandler and his associates have done a similar type of investigation involving some of the same strains which we studied (O. Kandler, personal communication). They are proposing to designate as a species a group corresponding to our homology group B-1. This species would be in immunological group I of Gasser. Electron micrographs have revealed that the cell widths of the two B-1 reference strains were only slightly more than one-half the width of the other reference strains (Johnson, unpublished

data). And finally, DNA homology group B-2 should also be considered a new species (again, unnamed at this time), and strain ATCC 33200 (=VPI 7960) should be designated the type strain. This species would also be in immunological group I.

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REPRINT REQUESTS

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