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Taxonomic Study of the *Lactobacillus acidophilus* Group, with Recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and Synonymy of *Lactobacillus acidophilus* Group A3 (Johnson et al. 1980) with the Type Strain of *Lactobacillus amylovorus* (Nakamura 1981)

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Biochemical properties and DNA-DNA reassociation studies of *Lactobacillus acidophilus* strains isolated from humans and animals indicate that these include six genomospecies. Two new species can be differentiated from the established species of the genus *Lactobacillus*: *L. gallinarum* sp. nov. (type strain, ATCC 33199) and *L. johnsonii* sp. nov. (type strain, ATCC 33200). Furthermore, it was clarified that *L. acidophilus* group A3 (Johnson et al. 1980) is synonymous with *L. amylovorus*.

Lactobacillus acidophilus was first isolated from infant feces by Moro in 1900, and he named it "*Bacillus acidophilus*." In 1929, Holland included "*Bacillus acidophilus*" in the genus *Lactobacillus*. Subsequently, this species was studied by phenotypic characteristics such as sugar fermentation, serological properties, formation of lactic acid dehydrogenase (1, 13, 19, 20, 22, 23, 25), G+C content (moles percent) of DNA (6), and DNA-DNA hybridization (4). In 1970, a neotype strain was designated by Hansen and Mocquot (7). In 1980, Johnson et al. (9), Sarra et al. (21), and Lauer et al. (11) reported that the *Lactobacillus* strains identified as *L. acidophilus* by biochemical characteristics such as carbohydrate fermentation pattern and isomer of lactic acid were divided into several groups at the species level by DNA-DNA homology. These reports suggested that physiological properties, such as sugar fermentation and growth characteristics, were not adequate to differentiate the genospecies that had been delineated by DNA reassociation studies.

At present, three homology groups in the *L. acidophilus* group have been proposed as valid species, *L. acidophilus* (6), *L. crispatus* (2), and *L. gasseri* (12). However, no definitive statement on the nomenclature of the other *L. acidophilus* groups (Johnson et al. groups A3, A4, and B2 and Lauer et al. groups I-b, I-d, and II-b) has yet been published. In this study, we reexamined the phenotypic and genotypic characteristics of representative strains of the *L. acidophilus* group.

MATERIALS AND METHODS

Bacterial strains. The organisms used in this study are listed in Table 1. Twenty-six strains were isolated in our laboratory, and the other strains were obtained from the American Type Culture Collection, Rockville, Md. (ATCC); the Japan Collection of Microorganisms, Saitama, RIKEN, Japan (JCM); M. E. Sharpe, National Institute for Research in Dairying, University of Reading, Reading, United Kingdom; and G. Reuter, Institut für Lebensmittelhygiene Fleischhygiene und Technologie, Freie Universität, Berlin, Germany. Many of the culture collection strains have been examined previously (9, 11).

Morphological observations. Cell morphological characteristics were examined by microscopy. Colonial observations of 2-day growth on the surface of BL (glucose-blood liver) agar (Eiken, Tokyo, Japan) plates incubated in "steel-wool jars" under 100% CO_2 at 37°C were made.

Phenotypic properties. Biochemical tests, such as fermentation of carbohydrates, aerobic growth reaction, motility, growth at the fixed temperature, and gas production from glucose, were performed by the methods of Mitsuoka (17) and were tube tests. Tolerance to NaCl was examined as follows: 5 μl of Briggs liver broth (17) overnight cultures were spotted on BL agar plates containing NaCl and incubated in "steel-wool jars" under 100% CO_2 at 37°C for 48 h. Analysis of fermentation end products in broth samples was done by the method of Holdeman et al. (8). Configuration of lactic acid was analyzed enzymatically by the modification of the method of Mattsson (16). Electrophoretic mobility of NAD-dependent L-(+)- and D-(−)-lactate dehydrogenase (LDH) was determined by a modification of the method of Davis (3). The gel contained 7.5% acrylamide, and electrode buffer (Tris, 1 g; diethyl barbituric acid, 5.25 g; water to 1,000 ml) was diluted 1:10 before use. Each sample was electrophoresed at 1 mA for 30 min. The actual distance of migration varied from run to run, so the L-(+)- or D-(−)-LDH of *L. gasseri* JCM 1131^T was used as a control in experiments, and the distance of its migration was assigned an arbitrary value of 1.00. The distances of migration of all other enzymes examined were expressed relative to this value.

DNA base composition and DNA-DNA hybridization. The

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TABLE 1. Sources of *Lactobacillus* strains tested

Strain	Obtained from:	Source	Other strain designation(s)
L. acidophilus			
ATCC 4356^T	ATCC	Pharynx, human	DSM 20079, VPI 6032
ATCC 4355	ATCC	Intestine, rat	VPI 0328
JCM 1023	JCM	Feces, rat	ATCC 832, VPI 11760-B
L. amylovorus			
JCM 1126^T	JCM	Cattle waste-corn fermentation	DSM 20531, ATCC 33620
KD16-3	Own isolate	Feces, human	
ATCC 33198	ATCC	Small intestine, pig	VPI 1754
PN-Ri-2-3	Own isolate	Feces, pig	
F81	Own isolate	Feces, calf	
P5-49	Own isolate	Feces, pig	
PND-5-1	Own isolate	Feces, pig	
PNB-6-1	Own isolate	Feces, pig	
L. crispatus			
JCM 1185^T	JCM	Unknown	ATCC 33820, VPI 3199, DSM 20584
Sp34	G. Reuter	Sputum, human	
F199	Own isolate	Feces, human	
A 11	M. E. Sharpe	Unknown	VPI 11761
A269-21	Own isolate	Feces, human	
F3	Own isolate	Feces, chicken	
F5	Own isolate	Feces, chicken	
F16	Own isolate	Feces, chicken	
T-91	Own isolate	Feces, chicken	
L. gallinarum			
ATCC 33199^T	ATCC	Crop, chicken	VPI 1294
T-50	Own isolate	Feces, chicken	
F41	Own isolate	Feces, chicken	
TFC1	Own isolate	Feces, chicken	
TFC2	Own isolate	Feces, chicken	
TFC3	Own isolate	Feces, chicken	
L. gasseri			
JCM 1131^T	JCM	Human	ATCC 33323, DSM 20243
I-36-1	Own isolate	Feces, human	
F164	G. Reuter	Feces, human	ATCC 19992, VPI 6033
JCM 5343	JCM	Unknown	ATCC 4963, VPI 0334
JCM 5344	JCM	Vaginal tract	ATCC 9857, VPI 11089
F191	Own isolate	Feces, human	
F192	Own isolate	Feces, human	
F193	Own isolate	Feces, human	
F194	Own isolate	Feces, human	
L. johnsonii			
ATCC 33200^T	ATCC	Blood, human	VPI 7960
Omniflora	G. Reuter	Pharmacological preparation	
CHN-22-1-10	Own isolate	Feces, chicken	
5F49	Own isolate	Feces, mouse	
F133	Own isolate	Feces, calf	
PN-Ri-2-4	Own isolate	Feces, pig	
ATCC 11506	ATCC	Unknown	VPI 11088
ATCC 332	ATCC	Unknown	VPI 0325

TABLE 2. Phenotypic characteristics of the strains tested[a]

Characteristic	*L. acidophilus*	*L. amylovorus*	*L. crispatus*	*L. gallinarum*	*L. gasseri*	*L. johnsonii*
Acid from:						
Ribose	0/3[b]	0/8	2/9	0/6	0/9	0/8
Cellobiose	3/3	5/8	9/9	6/6	9/9	8/8
Lactose	3/3	7/8	8/9	2/6	9/9	7/8
Trehalose	3/3	1/8	5/9	0/6	9/9	5/8
Melibiose	0/3	4/8	8/9	6/6	1[c]/9	4/8
Raffinose	2/3	4/8	9/9	6/6	0/9	4/8
Dextrin	0/3	8/8	8/9	6/6	9/9	7/8
Starch	0/3	3/8	2/9	1/6	2/9	1/8
Mannitol	0/3	1/8	4/9	0/6	0/9	0/8
Esculin	3/3	5/8	9/9	6/6	9/9	8/8
Amygdalin	3/3	5/8	9/9	6/6	9/9	8/8
Migration of NAD-dependent LDH[d]						
L-(+)	1.35 ± 0.01	1.14 ± 0.02	1.14 ± 0.02	1.14 ± 0.02	1.02 ± 0.02	1.02 ± 0.02
D-(−)	—[e]	—	—	—	1.00 ± 0.01[f]	1.17 ± 0.02
Tolerant to 4.0% NaCl	—	0/8	1[c]/9	6/6	7/8	8/8

[a] All strains positive in glucose, mannose, galactose, maltose, sucrose, fructose, and salicin and produce DL-lactic acid; all strains negative in arabinose, xylose, rhamnose, melezitose, sorbitol, catalase, gas from glucose, and growth at 15°C.
[b] Number of positive strains/number of strains tested.
[c] Weak reaction.
[d] Migration distance of NAD-dependent L(+)- or D(−)-LDH: *L. gasseri* JCM 1131^T = 1.00.
[e] —, not tested.
[f] Seven of eight strains tested had a migration distance of 1.00 ± 0.01, and one strain had a distance of 1.10.

DNA was isolated and purified by the method of Marmur (14) from cells grown for 10 h in Briggs liver broth with glucose (0.5% [wt/vol]) as the carbon source. Determination of moles percent G+C content was done by the thermal denaturation temperature by the method of Marmur and Doty (15). DNA base composition was calculated from the melting temperature (15), which was obtained with a spectrophotometer (model DU-8B; Beckman Instruments, Inc., Fullerton, Calif.). *Escherichia coli* DNA (Sigma) was used as the reference. DNA-DNA hybridization experiments were performed by the method of Johnson et al. (9). S1 nuclease treatment was performed as described previously (5). DNAs were labeled by nick translation with ^{3}H (New England Nuclear Corp., Boston, Mass.).

RESULTS AND DISCUSSION

All of the strains studied were facultatively anaerobic, gram-positive, non-spore-forming rods that produced DL-lactic acid from Briggs liver broth as the major fermentation product. Cells of the strains tested from BL agar plates cultures were short to long rods (0.5 to 1.5 μm in width and 1.5 to 10.0 μm in length) and occurred singly, in pairs, and sometimes in short chains. Surface colonies on BL agar plates were 0.5 to 2.0 mm in diameter, circular to slightly irregular, entire, grayish brown to brown, and rough. The other phenotypic properties of all strains are shown in Table 2. On the basis of the phenotypic characteristics, all strains used were identified as *L. acidophilus* group strains.

DNA-DNA homology results indicated that these *L. acidophilus* group strains were divided into six groups at the species level, and these data were in full agreement with the results obtained previously by Johnson et al. (9) and Lauer et al. (11) (Table 3). Our results confirm previous reports (9, 11) that the Johnson et al. groups A4 and B2 are distinct species. Further, we showed that the Johnson et al. group A3 strain 1754 (parent strain of ATCC 33198) was a member of the *L. amylovorus* DNA reassociation group. Thus, *L. acidophilus* group A3 (9) is synonymous with *L. amylovorus* Nakamura 1981 (18). It has been shown that, because phenotypic characteristics of *L. amylovorus* are not different from those of *L. acidophilus*, *L. crispatus*, and *L. gasseri*, the DNA-DNA homology test is necessary to differentiate *L. amylovorus* from other species (10).

Johnson et al. (9) and Wayne et al. (24) have indicated that it is necessary to find differential phenotypic criteria in order to designate new species. In the present study, migration of NAD-dependent L-(+)- and/or D-(−)-LDH and tolerance to 4% NaCl differentiated the two new species from other species of the *L. acidophilus* group, as shown in Table 3. The results of migration of NAD-dependent L-(+)- and D-(−)-LDH in this study were in full agreement with the results reported by Lauer et al. (11).

Description of *Lactobacillus gallinarum* sp. nov. *Lactobacillus gallinarum* (gallin.ar'um. L. gen. pl. *gallinarum* of hens). Facultatively anaerobic, gram-positive, non-spore-forming rods that produce DL-lactic acid as a major fermentation product from glucose. Cells of the strains from BL agar plate cultures are short to long rods (0.5 to 1.5 μm in width and 1.5 to 10.0 μm in length) and occur singly, in pairs, and sometimes in short chains. Surface colonies on BL agar plates are 0.5 to 2.0 mm in diameter, circular to slightly irregular, entire, grayish brown to reddish brown, and rough. Grown at 15°C. Catalase negative. Acid is produced without gas formation from glucose, mannose, maltose, galactose, sucrose, fructose, cellobiose, melibiose, raffinose, dextrin, esculin, salicin, and amygdalin. No acid formation from arabinose, xylose, rhamnose, ribose, trehalose, melezitose, mannitol, and sorbitol. Acid production from lactose and starch is variable. Strains are tolerant to 4.0% NaCl. The G+C content of the DNA is 35.9 to 37.2 mol%. Isolated from chicken intestine. Type strain is ATCC 33199.

Description of *Lactobacillus johnsonii* sp. nov. *Lactobacillus johnsonii* (john.so'ni.i. M.L. gen. n. *johnsonii* of

TABLE 3. DNA-DNA homology among the strains tested

Strain	G+C content (mol%)	[^{3}H]DNA from: ATCC 4356^T	P5-49	KD16-3	Sp34	T-50	JCM 1131^T	Omniflora	Homology group: Johnson et al.	Homology group: Lauer et al.
L. acidophilus										
ATCC 4356^T	32.3	100	—[a]	—	—	—	—	—	A-1	I-a
ATCC 4355	33.0	100	27	15	6	15	—	0	A-1	
JCM 1023	33.9	106	—	—	—	—	—	—	A-1	
L. amylovorus										
JCM 1126^T	38.0	—	104	77	—	—	—	—		
KD16-3	37.2	—	86	100	26	36	—	—		I-b
ATCC 33198	37.3	—	—	78	—	—	—	—	A-3	I-b
PN-Ri-2-3	36.8	15	—	107	12	—	23	0		
F81	36.9	—	—	81	—	—	—	—		
P5-49	35.8	—	100	—	—	—	—	—		
PND-5-1	36.0	—	106	—	—	—	—	—		
PNB-6-1	35.9	—	92	—	—	—	—	—		
L. crispatus										
JCM 1185^T	35.4	—	19	13	103	15	—	0		
Sp34	33.4	—	—	17	100	20	—	5		I-c
F199	—	8	—	17	69	18	4	6		
A269-21	32.5	—	—	—	56	—	—	7		
A 11	—	—	—	—	62	—	—	2	A-2	
F3	35.1	15	—	16	62	19	3	8		
F5	35.7	—	—	8	104	12	—	0		
F16	—	—	—	—	69	—	—	5		
T-91	—	1	—	—	60	—	18	—		I-c
L. gallinarum										
ATCC 33199^T	35.9	—	—	—	—	78	—	—	A-4	I-d
T-50	—	—	27	20	—	100	—	—		I-d
F41	37.0	7	—	18	—	83	2	—		
TFC1	36.9	—	—	—	—	80	—	—		
TFC2	37.2	—	—	—	—	69	—	—		
TFC3	36.2	—	—	—	—	75	—	—		
L. gasseri										
JCM 1131^T	32.9	7	8	6	6	0	100	30		II-a
I-36-1	—	4	—	7	5	6	58	36	B-1	II-a
F164	—	—	—	—	—	—	—	—	B-1	II-a
JCM 5343	33.1	—	—	—	—	—	72	—	B-1	
JCM 5344	34.0	—	—	—	—	—	70	—	B-1	
F191	32.7	2	—	—	—	—	61	—		
F192	32.5	—	—	—	—	—	60	—		
F193	32.8	—	—	—	—	—	69	—		
F194	33.0	—	—	—	—	—	59	—		
L. johnsonii										
ATCC 33200^T	33.0	—	7	—	—	—	—	69	B-2	
Omniflora	34.0	—	—	17	9	10	—	100		II-b
CHN-22-1-10	—	4	—	13	9	—	20	68		II-b
5F49	32.7	4	—	—	0	—	25	78		
F133	34.8	—	—	—	0	—	30	77		
PN-Ri-2-4	—	—	—	19	6	21	—	91		
ATCC 11506	32.5	—	—	—	10	—	—	70	B-2	
ATCC 332	33.1	—	—	—	—	—	—	79	B-2	

[a] —, not tested.

Johnson; named for J. L. Johnson, an American microbiologist). The following biochemical properties are different from those of *L. gallinarum*: acid from trehalose, melibiose, raffinose, and dextrin is variable. G+C content of DNAs is 32.7 to 34.8 mol%. Isolated from human blood and feces of chicken, mice, calf, and pig. Type strain is ATCC 33200.

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