Emended Descriptions of *Lactobacillus sake* (Katagiri, Kitahara, and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): Numerical Classification Revealed by Protein Fingerprinting and Identification Based on Biochemical Patterns and DNA-DNA Hybridizations

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A former subgenus of the genus *Lactobacillus*, “*Streptobacterium*,” comprises a wide range of species, including the so-called “atypical streptobacteria,” which includes the *Lactobacillus sake*-*Lactobacillus curvatus* group. Various identification systems and differentiation criteria for *L. sake* and *L. curvatus* have been described previously and are well established. The phenotypic diversity within these two species was the reason for comparing phenotypic variations with DNA homology data. Previously described biotypes of *L. sake* (Katagiri, Kitahara, and Fukami) and *L. curvatus* (Abo-Elnaga and Kandler) were the basis for selecting strains. Strains of all known biotypes of these species were examined to determine their biochemical reactions, their physiological growth characteristics, their total soluble protein contents as determined by a pattern analysis in which native polyacrylamide gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis with colloidal Coomassie blue and silver diamine staining were used, and their levels of DNA homology as determined by DNA-DNA hybridization experiments. All of the phenotypic analyses revealed a diversity within the taxa, whereas the DNA-DNA hybridization analysis revealed that the level of genomic homogeneity within each species was relatively high. The phenotypic diversity and genomic homogeneity which we observed allowed us to describe subgroups of *L. sake* and *L. curvatus*. The descriptions of *L. sake* and *L. curvatus* are emended accordingly. These subgroups which we describe may be the basis for defining subspecies within these two species.

The facultatively heterofermentative lactobacilli comprise a wide range of species, including species and subspecies of technological and clinical interest, such as *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus paracasei* subspecies. The so-called “atypical streptobacteria” (13, 18, 19, 34, 44), which are significant spoilage bacteria, are of special interest and occur in naturally or artificially fermented meat. The habitats of *Lactobacillus sake* and *Lactobacillus curvatus* are very diverse. These organisms are important in the production of fermented plant material (silage, sauerkraut, mixed pickles, etc.) and in the spoilage of plant material as well. They also occur in beverages (e.g., beer) and even in manure (14, 22). *L. sake* was first isolated from sake (rice wine) starter (22). Our strain collection also includes *L. sake* strains of human origin, a habitat not described previously for this species (Table 1). These bacteria have been called atypical streptobacteria compared with the “streptobacteria,” a term used in classical and descriptive taxonomy (31) which is no longer used in genomically based taxonomy (46). The ability to grow at 2°C and pH 3.9 was recognized as a main criterion for differentiation. Table 2 shows other distinguishing characteristics that are based on morphological and physiological data (31, 36, 37). *L. sake* and *L. curvatus* are atypical streptobacteria. Even though criteria for differentiating these

two species are well established, the phenotypic diversity within each species is obvious (12, 39, 41, 42). The strains of a third species, “*Lactobacillus bavaricus*,” which initially was isolated from sauerkraut (48), were recently identified as members of either *L. sake* or (in the case of one strain) *L. curvatus* (21).

Various identification systems have been proposed and used to differentiate atypical streptobacteria and related organisms, which reflects the remarkable phenotypic diversity of this group (12, 14, 16, 28, 35, 39, 41–44). A number of workers have used advanced computer technology to perform numerical taxonomy analyses of lactobacilli obtained from meat and meat products (2, 10, 11, 15, 45). Champomier et al. (3) identified approximately 15 different biochemical patterns for strains that were genomically identified as *L. sake* isolates by DNA-DNA hybridization studies. Slight differences in the patterns of fermentation of carbohydrates by *L. sake* isolates obtained from radurized meat have also been described (29).

Five groups of *L. sake* and *L. curvatus* fermentation patterns were recognized in a recent study of the microflora isolated from naturally fermented Greek dry salami (40). The fermentation of melibiose and arginine hydrolysis (*L. curvatus* is negative in both tests) were regarded as the main criteria that differentiate *L. sake* and *L. curvatus*. Within the two groups of *L. sake* the fermentation of trehalose was considered an important reaction, and within the two groups of *L. curvatus* the fermentation of sucrose seemed to be a criterion for differentiation. One group was referred to as an *L. sake*-*L. curvatus* mixed group because the strains belonging to this group were

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TABLE 1. Strains used^a

Taxon	Strain	Other designation(s)	Source	Comment(s) or supplier
<i>L. sake</i> subgroup I	DSM 20017 ^T	ATCC 15521 ^T , NCFB 2714 ^T	Starter (rice wine)	DSM
	R 61b		Raw sausage	Isolated by G. Reuter
	R 41d		Raw sausage	Isolated by G. Reuter
	Rv 2f/1a		Raw sausage	Isolated by G. Reuter
	Rv 2f/1b		Raw sausage	Isolated by G. Reuter
	R 14a/a		Raw sausage	Isolated by G. Reuter
<i>L. sake</i> subgroup II	Nr. 440/2151	R 14b/a	Meat	W. H. Holzapfel, BFE
	CCUG 31331		Raw sausage	DSM
	R 14b/b		Raw sausage	Isolated by G. Reuter
	R 28d/a		Raw sausage	Isolated by G. Reuter
	R 28d/b		Raw sausage	Isolated by G. Reuter
	R 6a/a		Raw sausage	Isolated by G. Reuter
	R 6a/b		Raw sausage	Isolated by G. Reuter
	R 116/1a		Raw sausage	Isolated by G. Reuter
	W 8 N		Sliced meat product	Isolated by G. Reuter
	Nr. 438/2087		Meat	W. H. Holzapfel, BFE
	Nr. 439/2154		Meat	W. H. Holzapfel, BFE
	FKS-II-15 Nr. 556		Salad with herring	W. H. Holzapfel, BFE
	FKS-II-16 Nr. 557		Salad with herring	W. H. Holzapfel, BFE
	CCUG 8045		Human	CCUG
	CCUG 30939		Human	CCUG
	CCUG 32077		Human	CCUG
	CCUG 32584		Human	CCUG
<i>L. curvatus</i> subgroup I	DSM 20019 ^T	ATCC 25601 ^T , NCFB 2739 ^T	Milk	DSM
	W 55d		Sliced meat product	Isolated by G. Reuter
	W 3		Sliced meat product	Isolated by G. Reuter
	R 57		Raw sausage	Isolated by G. Reuter
	R 111i		Raw sausage	Isolated by G. Reuter
	R 111j		Raw sausage	Isolated by G. Reuter
	R 28c		Raw sausage	Isolated by G. Reuter
	R 27c/b		Raw sausage	Isolated by G. Reuter
	R 60a		Raw sausage	Isolated by G. Reuter
	Rv 2eI		Raw sausage	Isolated by G. Reuter
	CCUG 31333		Raw sausage	Isolated by G. Reuter
	CCUG 31332		Retail market	Isolated by G. Reuter
	R 28/1a		Raw sausage	Isolated by G. Reuter
	153/1f		Raw sausage	Isolated by G. Reuter
<i>L. curvatus</i> subgroup II	W 21N	Rv 40a Rei 23/1aII	Sliced meat product	Isolated by G. Reuter; formerly " <i>L. bavaricus</i> "
	CCUG 34545		Raw sausage	Isolated by G. Reuter
	W 54d		Raw sausage	Isolated by G. Reuter
	R 114a		Raw sausage	Isolated by G. Reuter
	R 17b		Raw sausage	Isolated by G. Reuter
	R 7g/a		Raw sausage	Isolated by G. Reuter
	R 7g/b		Raw sausage	Isolated by G. Reuter
	Rv 2b/b		Raw sausage	Isolated by G. Reuter
	R 116/3a		Raw sausage	Isolated by G. Reuter
	R 116/3b		Raw sausage	Isolated by G. Reuter
	R 111g		Raw sausage	Isolated by G. Reuter
	R 102b		Raw sausage	Isolated by G. Reuter
	R 105e/b		Raw sausage	Isolated by G. Reuter
<i>L. casei</i>	ATCC 393 ^T	DSM 20011 ^T	Cheese	ATCC
<i>L. rhamnosus</i>	DSM 20021 ^T	ATCC 7469 ^T		DSM
<i>L. paracasei</i> subsp. <i>paracasei</i>	DSM 5622 ^T	ATCC 27216	Saliva	DSM
	DSM 20020			DSM
<i>L. alimentarius</i>	DSM 20249 ^T	R 13 ^T	Marinated fish product	Isolated by G. Reuter ^b
<i>L. farciminis</i>	DSM 20184 ^T	Rv 4na ^T	Raw sausage	Isolated by G. Reuter ^b
<i>L. graminis</i>	CCUG 32238 ^T	DSM 20719 ^T	Grass silage	CCUG
<i>L. murinus</i>	CCUG 33904 ^T	DSM 20452 ^T	Rat digestive tract	CCUG
<i>L. acidophilus</i>	DSM 20079 ^T	ATCC 4356 ^T	Human	DSM
	ATCC 314			ATCC

Continued on following page

TABLE 1—Continued

Taxon	Strain	Other designation(s)	Source	Comment(s) or supplier
<i>L. gasseri</i>	DSM 20243 ^T	ATCC 33323 ^T	Human	DSM
<i>L. crispatus</i>	DSM 20584 ^T	ATCC 33820 ^T	Eye	DSM

^a Information was obtained from the suppliers. Abbreviations: CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; BFE, Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, Md.; NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

^b See reference 38.

arginine positive and melibiose negative. The phenotypic diversity of *L. sake* and *L. curvatus* was the reason for comparing phenotypic data with DNA homology data in this study.

MATERIALS AND METHODS

Bacterial strains. The strains included in this study are listed in Table 1. Previously described biotypes of *L. sake* and *L. curvatus*, as defined by Reuter (35, 39), were the basis for selecting representative strains. The characteristics of these biotypes are shown in Table 3. More than 50 strains of all known biotypes of these species were examined. Most of these strains were isolated from meat and meat products and were kept in the culture collection of the Institute of Meat Hygiene and Technology of the Free University of Berlin. *L. casei* ATCC 393^T (T = type strain), *L. rhamnosus* DSM 20021^T, *L. paracasei* subsp. *paracasei* DSM 20020 and DSM 5622^T, *Lactobacillus alimentarius* DSM 20249^T, *Lactobacillus farciminius* DSM 20184^T, *Lactobacillus graminis* CCUG 32238^T, *Lactobacillus murinus* CCUG 33904^T, *Lactobacillus acidophilus* ATCC 4356^T and ATCC 314, *Lactobacillus gasseri* DSM 20243^T, and *Lactobacillus crispatus* DSM 20584^T were also included in this study to determine their positions within the genus *Lactobacillus*.

Biochemical tests. Biochemical properties, such as fermentation of carbohydrates, gas production from glucose, and growth at defined temperatures, were determined by using the classical macrotube tests described by Lerche and Reuter (26) and Reuter (33, 35) for all strains. A total of 21 carbon substrates were used in acidification tests in a semisolid medium (25, 26); results were determined after incubation for 6 days. Growth at defined temperatures was determined after incubation for 3 days at 4 ± 0.1°C and for 1 day at 42 ± 0.1°C.

The carbohydrates and derivatives tested were L-(+)-arabinose (catalog no. 1492; Merck), D-(+)-glucose (catalog no. 8342; Merck), lactose (catalog no. 7657; Merck), D-(+)-sucrose (catalog no. 7651; Merck), D-(+)-maltose (catalog no. 5910; Merck), D-(+)-trehalose (catalog no. 8353; Merck), D-(+)-melibiose (catalog no. 12240; Merck), D-(+)-cellobiose (catalog no. 2352; Merck), D-(+)-raffinose (melitose) (catalog no. 7549; Merck), D-(+)-mannitol (catalog no. 5982; Merck), D-(+)-salicin [2-O-(β-D-glucopyranosido)-benzylalcohol] (catalog no. 7665; Merck), L-(+)-rhamnose (catalog no. 4736; Merck), D-(+)-xylose (catalog no. 8692; Merck), D-(+)-mannose (catalog no. 5984; Merck), D-(+)-melezitose (catalog no. 28550; Serva), myo-inositol (catalog no. 4728; Merck), D-(+)-sorbitol (catalog no. 7758; Merck), D-(+)-inulin (catalog no. 4733; Merck), dextrin (catalog no. 3006; Merck), D-(+)-galactose (catalog no. 4062; Merck), D-(+)-fructose (catalog no. 5323; Merck), D-(+)-ribose (catalog no. 7605; Merck), L-arginine (catalog no. 1542; Merck), amygdalin (catalog no. 10050; Fluka), and sodium gluconate (catalog no. 822058; Merck).

The production of slime from sucrose (dextran formation), the production of hydrogen peroxide, the production of acetoin (3-hydroxy-2-butanone) from glucose, the production of NH₃ from arginine, the production of lactic acid, and growth in the presence of 10% NaCl were determined by the methods described by Reuter (35). The configuration and amount of lactic acid were determined enzymatically by using D-lactate and L-lactate dehydrogenase (catalog no. 1112821; Boehringer Mannheim GmbH, Mannheim, Germany).

DNA base composition. Cultures were grown in MRS broth (7) for 48 h at 30°C and were harvested by centrifugation. DNA was isolated and purified by using a previously described technique (9). The DNA was diluted to a concentration of 20 µg/ml in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0). Samples were analyzed as described by Dicks et al. (9), and the melting temperature was calculated from the average of three determinations. The DNA base composition (guanine-plus-cytosine [G+C] content) was calculated by using the equation of De Ley (4).

DNA-DNA hybridization. Cultures were grown in MRS broth (7) for 48 h at 30°C. The medium used for labeling DNA was MRS broth supplemented with [¹⁴C]uracil and [¹⁴C]adenine, and the method used was the method described by Dellaglio et al. (5, 6). Reassociation was performed in 2× SSC in a water bath at 55.8°C (30°C lower than the melting temperature). Percentages of DNA homology were calculated by using the single-point competition technique (20). The strains used for DNA-DNA hybridization experiments are listed in Tables 4 and 5.

Protein analysis. Three protein electrophoresis methods were used by workers in three independent laboratories. The strains used in the three analyses are listed in Table 5.

(i) **Numerical analysis of total soluble cell protein patterns (native polyacrylamide gel electrophoresis [PAGE]).** Strains were cultured in MRS broth (7). The methods used for isolation of total soluble cell proteins, protein concentration determination, electrophoresis, densitometry, normalization of densitometric traces, and conversion of densitometric traces were the methods described by Dicks et al. (8, 9). The Pearson product-moment correlation coefficients (*r*) between pairs of densitometric traces were calculated for 51 gels (three gels per strain). Clustering was done by the unweighted average pair group method.

(ii) **Numerical analysis of total soluble cytoplasmatic protein patterns with silver staining (semiautomated analysis performed with minigels).** Bacterial cells were incubated overnight in 18 ml of MRS broth (7) at 30°C. The methods used for protein purification, protein concentration determination, electrophoresis, densitometry, normalization of densitometric traces, and conversion of densitometric traces were the methods described by Klein et al. (23, 24). The special procedures used are described below. Each protein sample was diluted to a standard concentration of 0.4 µg/µl. Samples were electrophoresed on gradient sodium dodecyl sulfate (SDS)-polyacrylamide minigels (concentration of polyacrylamide, [Pharmacia, Uppsala, Sweden], 10 to 15%) by using a semiautomated system (Phast-System; Pharmacia). Protein standards (low-molecular-weight calibration kit; Pharmacia) having known molecular weights were included in each gel as internal marker proteins. Two samples of the proteins of each strain were tested on the same gel; one sample contained the protein markers, and one sample did not. This allowed us to make comparisons within and between gels and to calculate the molecular weights of the sample protein bands. Staining was performed by using the diamine silver staining method as recommended by the manufacturer (Pharmacia). Since silver diamine is not stable, solutions had to be prepared just before use. The processing temperatures were kept below 20°C. The stained protein patterns were scanned with a Hirschmann model elscript 400 optical densitometer and digitized. The levels of similarity between profiles (averages of three separate electrophoretic runs) were determined by using *r*, and clustering was performed by the unweighted pair

TABLE 2. Characteristics of streptobacteria and atypical streptobacteria^a

Taxon	Growth on solid media						Growth in liquid media			
	Plate count agar			Media for lactobacilli			Minimum temp (°C) after 10 to 12 days	Maximum temp (°C) after 4 to 6 days	Final pH after 5 days at 30°C	Growth at pH 3.9
	Growth rate	Colony size	Cell shape	Growth rate	Colony diam (mm)	Cell shape				
Streptobacteria	Reduced	Pinpoints	Rod	Not reduced	1–2	Rods	4–8	42–45	3.7–3.8	+
Atypical streptobacteria	Not markedly reduced	Small	Coccoid	Not reduced	0.5–1	Short rods	2–4	40–42	3.9–4.1	–

^a Data from references 36 and 37.

TABLE 3. Criteria for differentiating *L. sake* and *L. curvatus* biotypes isolated from meat products^a

Taxon	Biotype(s)	Production of acid from ^b :											Formation of H ₂ O ₂ ^c	Growth in the presence of 10% NaCl ^c	Production of NH ₃ from arginine ^c	Production of acetoin from glucose ^c	Configuration of lactic acid
		Arabinose	Melibiose	Sucrose	Maltose	Lactose	Trehalose	Cellobiose	Salicin	Amygdalin	Gluconate	Dextran					
<i>L. sake</i> subgroups	C 1, C 2, C 3	+	+	+	v	—	v	v	v	—	(+)	v	—	+	+	+	DL
	A 1	—	+	+	v	—	+	v	+	—	(+)	v	—	+	+	+	DL
<i>L. curvatus</i> subgroup I	A 3	—	—	+	+	v	+	—	—	—	—	—	+	—	—	—	DL
	A 5	—	—	+	+	+	+	+	+	+	—	+	+	—	—	—	DL
	A 6, A 7	—	—	+	+	—	—	v	v	+	—	v	+	—	—	—	DL
	A 4, A 8	—	—	—	+	v	—	v	—	—	—	—	+	—	—	—	DL
	C 2a ^d	(+)	+	(+)	(+)	(+)	(+)	+	(+)	—	(+)	—	—	(+)	+	(+)	L
<i>L. curvatus</i> subgroup II	A 2	—	+	+	—	—	+	v	v	—	(+)	—	—	(+)	v	—	DL

^a Data from references 35 and 39 and this study.^b +, positive (pH < 4.6); (+), weakly positive (pH 4.6 to 5.0); —, negative (pH > 5.0); v, variable. The initial pH was 6.5 to 6.8.^c +, positive; (+), weakly positive; —, negative; v, variable.^d Formerly "*L. bavaricus*," an organism that is not biochemically typical of *L. sake* and *L. curvatus*.TABLE 4. DNA-DNA hybridization data and phenotypic characteristics for *L. sake* and *L. curvatus* strains

Taxon	Strain	% DNA-DNA homology with:		Biotype ^a	Production of acid from ^b :											Formation of H ₂ O ₂ ^c	Growth in the presence of 10% NaCl ^c	Production of NH ₃ from arginine ^c	Production of acetoin from glucose ^c	Configuration of lactic acid	Growth at ^c :	
		<i>L. sake</i> DSM 20019 ^T	<i>L. curvatus</i> DSM 20017 ^T		Arabinose	Melibiose	Sucrose	Maltose	Lactose	Trehalose	Cellobiose	Salicin	Amygdalin	Gluconate	Dextran						4°C	42°C
<i>L. sake</i> subgroup I	DSM 20017 ^T	100	39	(+)	+	+	+	+	(+)	(+)	—	(+)	—	(+)	ND ^d	ND	ND	ND	ND	ND	ND	ND
	R 41d	84	45	C 2	+	+	+	(+)	—	+	—	—	—	(+)	—	—	+	+	(+)	DL	+	—
	R 61b	98	46	A 1	—	+	+	—	—	+	—	+	—	(+)	—	—	(+)	+	(+)	DL	+	+
<i>L. sake</i> subgroup II	CCUG 31331	92	54	C 3	+	+	+	+	—	+	—	—	—	(+)	—	—	+	+	+	DL	(+)	+
	R 116/1a	98	51	C 1	+	+	+	—	—	—	(+)	(+)	—	(+)	+	—	+	+	(+)	DL	(+)	(+)
<i>L. curvatus</i> subgroup I	DSM 20019 ^T	45	100	A 8	—	—	—	+	—	—	+	—	—	—	ND	ND	ND	ND	ND	ND	ND	ND
	CCUG 31332	49	102	A 3	—	—	+	+	+	+	—	—	—	—	—	+	—	—	—	DL	(+)	(+)
	R 57	40	102	A 3	—	—	+	+	—	+	(+)	—	—	—	—	ND	—	—	—	DL	ND	ND
	R 100	51	94	A 3	—	—	+	+	—	+	—	—	—	—	—	(+)	—	—	—	DL	(+)	(+)
	W 55d	40	89	A 5	—	—	(+)	(+)	+	+	+	+	+	—	+	(+)	—	—	—	DL	(+)	(+)
	R 28c	41	100	A 5	—	—	(+)	(+)	(+)	+	+	(+)	+	—	+	+	—	—	—	DL	(+)	(+)
	R 111i	46	80	A 6	—	—	(+)	(+)	—	—	+	(+)	+	—	—	+	—	—	—	DL	(+)	(+)
	Rv 2eI	40	89	A 6	—	—	(+)	(+)	—	—	(+)	—	+	—	+	+	—	—	—	DL	(+)	(+)
	R 111j	43	92	A 6	—	—	+	+	—	—	(+)	(+)	+	—	+	+	—	—	—	DL	(+)	(+)
	R 60a	39	87	A 7	—	—	+	+	—	—	—	—	+	—	—	+	—	—	—	DL	+	(+)
	CCUG 31333	45	96	A 8	—	—	—	+	+	—	—	—	—	—	—	+	—	—	—	DL	+	(+)
	Rv 4c	42	101	A 4	—	—	—	+	—	—	(+)	—	—	—	—	(+)	—	—	—	DL	(+)	—
<i>L. curvatus</i> subgroup II	W 21N ^e	40	91	C 2a	(+)	+	(+)	(+)	(+)	(+)	+	(+)	—	(+)	—	—	(+)	+	(+)	L	(+)	+
	CCUG 34545	50	84	A 2	—	+	+	—	—	+	(+)	—	—	(+)	—	—	(+)	—	—	DL	(+)	(+)
	W 54d	48	101	A 2	—	+	+	—	—	+	—	+	—	(+)	—	—	(+)	+	—	DL	+	—

^a See Table 3.^b +, positive (pH < 4.6); (+), weakly positive (pH 4.6 to 5.0); —, negative (pH > 5.0). The initial pH was 6.5 to 6.8.^c +, positive; (+), weakly positive; —, negative.^d ND, not determined.^e Formerly "*L. bavaricus*," an organism that is not biochemically typical of *L. sake* and *L. curvatus*.

TABLE 5. Results of biochemical, protein fingerprinting, and DNA-DNA hybridization experiments

Taxon	Strain	Biotype ^a	Protein group as determined by:			DNA homology group ^b
			Native PAGE	SDS-PAGE with silver staining	SDS-PAGE with Coomassie blue staining	
<i>L. sake</i> subgroup I	DSM 20017 ^T	C	1	S 1	S I	Sake
	R 61b	A 1	1	S 1	S I	Sake
	R 41d	C 2	1	S 1	S I	Sake
	Rv 2f/1a	C			S I	
	Rv 2f/1b	C			S I	
	R 14a/a	C			S I	
	Nr. 440/2151	C			S I	
<i>L. sake</i> subgroup II	CCUG 31331	C 3		S 2	S II	Sake
	R 14b/a	C 3	1	S 2	S II	Sake
	R 14b/b	C			S II	
	R 28d/a	A 1			S II	
	R 28d/b	A 1			S II	
	R 6a/a	A 1			S II	
	R 6a/b	A 1			S II	
	R 116/1a	C 1	1	S 2	S II	Sake
	W 8 N	A 1			S II	
	Nr. 438/2087	C 2			S II	
	Nr. 439/2154	A 1			S II	
	FKS-II-15 Nr. 556	C 3			S II	
	FKS-II-16 Nr. 557	C 2			S II	
	CCUG 8045	C			S II	
	CCUG 30939	C			S II	
	CCUG 32077	C		S 2	S II	
	CCUG 32584	C			S II	
<i>L. curvatus</i> subgroup I	DSM 20019 ^T	A 8	2		C I	Curvatus
	W 55d	A 5	2		C I	Curvatus
	W 3	A 4			C I	
	R 111i	A 6	2	C 1	C I	Curvatus
	R 111j	A 6	2	C 1	C I	Curvatus
	R 28c	A 5		C 1	C I	
	R 27c/b	A 4			C I	
	R 57	A 3	2		C I	Curvatus
	R 60a	A 7	2	C 1	C I	Curvatus
	Rv 2eI	A 6	2	C 1	C I	Curvatus
	Rv 40a	A 8	2	C 1	C I	Curvatus
	Rei 23/1aII	A 3	2	C 1	C I	Curvatus
	CCUG 31332	A 3			C I	Curvatus
	R 28/1a	A 4			C I	
	153/1f	A 5			C I	
	W 21N	C 2a			C I	Curvatus
<i>L. curvatus</i> subgroup II	CCUG 34545	A 2			C II	Curvatus
	R 60	A 2	3	C 2	C II	Curvatus
	R 17b	A 2			C II	
	W 54d	A 2	3	C 2	C II	Curvatus
	R 7g/a	A 2			C II	
	R 7g/b	A 2			C II	
	Rv 2b/b	A 2		C 3	C II	
	R 116/3a	A 2		C 3		
	R 116/3b	A 2		C 3	C II	
	R 111g	A 2			C II	
	R 102b	A 2		C 3	C II	
	R 105c/b	A 2			C II	
<i>L. casei</i>	ATCC 393 ^T	NA ^c	Other	Other	Other	Other
<i>L. rhamnosus</i>	DSM 20021 ^T	NA	Other	Other	Other	Other
<i>L. paracasei</i> subsp. <i>paracasei</i>	DSM 5622 ^T	NA	Other	Other	Other	Other
	DSM 20020	NA	Other	Other	Other	Other
<i>L. alimentarius</i>	DSM 20249 ^T	NA	Other	Other	Other	Other
<i>L. farciminis</i>	DSM 20184 ^T	NA	Other	Other	Other	Other

Continued on following page

TABLE 5—Continued

Taxon	Strain	Biotype ^a	Protein group as determined by:			DNA homology group ^b
			Native PAGE	SDS-PAGE with silver staining	SDS-PAGE with Coomassie blue staining	
<i>L. graminis</i>	CCUG 32238 ^T	NA	Other	Other	Other	Other
<i>L. murinus</i>	CCUG 33904 ^T	NA	Other	Other	Other	Other
<i>L. acidophilus</i>	DSM 20079 ^T	NA	Other	Other	Other	Other
	ATCC 314	NA	Other	Other	Other	Other
<i>L. gasseri</i>	DSM 20243 ^T	NA	Other	Other	Other	Other
<i>L. crispatus</i>	DSM 20584 ^T	NA	Other	Other	Other	Other

^a See Table 3.^b Members of DNA homology group Sake exhibit high levels of homology (>80%) with the type strain of *L. sake*, and members of DNA homology group Curvatus exhibit high levels of homology (>80%) with the type strain of *L. curvatus*.^c NA, not applicable.

group method using average linkage and the single-link cluster analysis method (both in NTSYS, version 1.60).

(iii) **Numerical analysis of the total soluble cytoplasmatic protein patterns with colloidal Coomassie blue (Novex gels).** The methods used to grow strains, purify proteins, and determine protein concentrations were the methods described by Klein et al. (23, 24) except as described below (32). Cells were harvested by rinsing two agar plates with 5 ml of 0.9% NaCl. The protein samples were diluted to a standard concentration of 1.0 µg/µl. Electrophoresis was performed with Tris-glycine gradient gels (polyacrylamide concentration, 8 to 16%; 15 traces; Novex) in a Novex Xcell apparatus (three gels per strain) with markers (Mark12; Novex) in positions 1, 4, 8, 12, and 15. The proteins were stained with colloidal Coomassie blue (Novex), and the gels were dried with a special drying solution (Anarapid; Anamed, Offenbach, Germany) between two sheets of cellophane. The stained protein patterns were scanned with a 1,200-dots-per-inch Highscreen Flatbed Colour IIs laser scanner) and digitized. Normalization of densitometric traces with background subtraction (rolling disk) and conversion were done with GelCompar, version 3.1 (Applied Maths, Kortrijk, Belgium). Clustering was performed by the unweighted average pair group method with the "none alignment" option.

RESULTS

On the basis of the biochemical reaction results and the results of the protein pattern analysis, each species could be divided into two subgroups. The subgroups of *L. sake* could not be differentiated by biochemical data, but could be distinguished by clear-cut differences in the protein cluster data. The type strain was included in subgroup I. The subgroups of *L. curvatus* could be distinguished by both biochemical test data and protein analysis data. Strains belonging to the protein cluster which included the type strain were melibiose negative and were assigned to subgroup I. The strains belonging to the second protein cluster fermented melibiose and were assigned to subgroup II. More detailed results are described below.

Biochemical tests. The biochemical reactions and physiological growth characteristics which we determined revealed that there were 13 different patterns, which corresponded to biotypes described by Reuter (35, 39), with some slight modifications (Table 3). Table 4 shows the results obtained for representative strains that were used in DNA hybridization experiments.

DNA base composition and DNA-DNA hybridization. The overall DNA G+C contents of the strains belonging to the *L. sake*-*L. curvatus* group ranged from 42 to 44 mol%. The levels of homology within *L. sake* ranged from 84 to 98%, and the levels of homology within *L. curvatus* ranged from 84 to 102%. The differences between the melting temperature of a homol-

ogous reaction and the melting temperature of a heterologous reaction for all strains were less than 5°C. Detailed data are shown in Table 4.

Protein analysis. Several clusters were distinguished on the basis of the results obtained with the different methods used for protein analysis and staining. The first method, native PAGE analysis, resulted in differentiation of *L. sake* and *L. curvatus* at an *r* level of 0.55 and in subdivision of *L. curvatus* into two clusters (Fig. 1) at an *r* level of 0.58. The second method (SDS-PAGE with silver staining) was the most sensitive method and resulted in strain-specific fingerprints and differentiation of five clusters (Fig. 2 and 3). The two *L. sake* clusters were differentiated at an *r* level of 0.75. The three *L. curvatus* clusters were differentiated at *r* levels of 0.78 and 0.81 for *L. curvatus* subgroup II and 0.91 for *L. curvatus* subgroup I. Almost all of the strains were investigated by using the third method (SDS-PAGE with colloidal Coomassie blue staining) and this analysis resulted in identification of four clusters. The species *L. sake* (Fig. 4) was subdivided into two clusters at an *r* level of 0.61, and the species *L. curvatus* (Fig. 5) was also subdivided into two clusters, at an *r* level of 0.72. All reference

FIG. 1. Dendrogram of strains of the *L. sake*-*L. curvatus* group derived from unweighted pair group average linkage of *r* values for the protein patterns revealed by native PAGE. The asterisks indicate strains for which designations are given in Table 1.

FIG. 2. Dendrogram of strains of the *L. sake* subgroups derived from unweighted pair group average linkage of *r* values for the protein patterns revealed by SDS-PAGE with silver diamine staining. The asterisk indicates a strain for which another designation is given in Table 1.

strains belonging to the related species listed in Table 1 clustered separately.

DISCUSSION

The strains of *L. sake* and *L. curvatus* which we used were selected on the basis of the biotypes described by Reuter (35, 39), because these biotypes reflect the considerable phenotypic diversity within the two species. Most strains originated from meat or meat products, which are the most important habitats. In addition, strains isolated from other sources (i.e., humans and salad with herring) were included.

In most cases biochemical reaction data allowed us to differentiate the two species. In addition, biochemical reaction data could be used to distinguish the *L. curvatus* subgroups. The *L. curvatus* subgroups were consistent with different biotypes (39) and protein fingerprinting results. The subgroups of *L. sake* could not be distinguished by biochemical reaction data, but the protein fingerprinting analysis revealed clearly separated clusters.

The differences within *L. sake* revealed by biochemical tests could not be used to subdivide this taxon. Some problems arose with biotype A 1 strains. These strains fermented melibiose but not arabinose and thus belonged to *L. curvatus* subgroup II on the basis of biochemical data. One strain belonging to biotype A 1, strain R 61b, was investigated by performing DNA-DNA hybridization experiments, and this strain was clearly identified as an *L. sake* strain (Table 4). This finding is consistent with the results of Samelis et al. (40), who described *L. sake* strains which had the unusual characteristic of not

FIG. 3. Dendrogram of strains of the *L. curvatus* subgroups derived from unweighted pair group average linkage of *r* values for the protein patterns revealed by SDS-PAGE with silver diamine staining. The asterisks indicate strains for which other designations are given in Table 1.

FIG. 4. Dendrogram of strains of the *L. sake* subgroups derived from unweighted pair group average linkage of *r* values for the protein patterns revealed by SDS-PAGE with colloidal Coomassie blue staining.

fermenting arabinose. The level of DNA relatedness of strain R 61b to *L. sake* was the reason for identifying biotype A 1 strains as *L. sake* strains and not as *L. curvatus* subgroup II strains. On the basis of protein data biotype A 1 strains were identified as *L. sake* subgroup II strains, whereas strain R 61b was identified as an *L. sake* subgroup I strain on the basis of protein data. Therefore, it is evident that biochemical data could not be the basis for subdivision of *L. sake*. Correct identification of biotype A 1 strains as members of *L. sake* required DNA homology studies or protein analysis experiments (especially native PAGE).

Within the *L. curvatus* subgroups differentiation on the basis of biochemical patterns was obvious. *L. curvatus* subgroup I strains were always arabinose negative and melibiose negative, and *L. curvatus* subgroup II strains were arabinose negative but melibiose positive. The carbon substrate fermentation patterns of the *L. curvatus* strains which we investigated corresponded fairly well with previously published data, but there were some

FIG. 5. Dendrogram of strains of the *L. curvatus* subgroups derived from unweighted pair group average linkage of *r* values for the protein patterns revealed by SDS-PAGE with colloidal Coomassie blue staining.

significant anomalies. We found that a number of *L. curvatus* subgroup I strains fermented trehalose, which was consistent with the findings of Nissen and Dainty (30); in contrast, Kandler and Weiss (22) found that these strains did not ferment trehalose. The data of Kandler and Weiss (22) corresponded well with our data for the type strain of *L. curvatus*. We found that all *L. curvatus* subgroup II strains fermented melibiose, while both Schillinger and Lücke (43) and Montel et al. (27) reported that *L. curvatus* is negative for this characteristic and *L. sake* is positive. Once again, our results were in agreement with the findings of Nissen and Dainty (30), who identified their strains as members of *L. sake* or *L. curvatus* by using a 23S rRNA probe. Therefore, we placed the melibiose-positive *L. curvatus* strains in a separate subgroup.

Our DNA-DNA hybridization experiments revealed high levels of relatedness within each species, in contrast to subgroups based on biochemical or protein patterns. The very high levels of homology within *L. sake* (84 to 98%) and within *L. curvatus* (84 to 102%) supported the conclusion that *L. sake* and *L. curvatus* are well-defined species. There is no reason to propose new species on the basis of differences in phenotypes. This conclusion is consistent with the conclusion of Stackebrandt and Goebel (47), who stated that the strains of a species could exhibit high levels of DNA relatedness despite significant differences in their phenotypes. The levels of homology between the *L. sake* strains and the type strain of *L. curvatus* were 39 to 54%, and the levels of homology between the *L. curvatus* strains and the type strain of *L. sake* were 39 to 51%. One strain, strain W 21N, which was formerly listed as an "*L. bavaricus*" strain, was identified as an *L. curvatus* strain. This strain was atypical in many respects when the biochemical reactions of *L. curvatus* and *L. sake* were examined. A level of DNA relatedness to *L. curvatus* of 91% and clustering in *L. curvatus* subgroup I by protein fingerprinting allowed us to identify this organism as an *L. curvatus* strain. This supported the conclusion that "*L. bavaricus*" is not a separate species (21). Kagermeier-Callaway and Lauer (21) identified most of the former "*L. bavaricus*" strains as members of *L. sake* by performing DNA homology studies. One strain (strain Tro-8/78) exhibited a level of DNA relatedness of 100% to *L. curvatus* DSM 20010, which exhibited a level of DNA homology of 100% to *L. curvatus* DSM 20019^T (6). We concluded that strain W 21N is another former "*L. bavaricus*" strain that exhibits a high level of DNA relatedness to the type strain of *L. curvatus*.

We analyzed the total soluble cytoplasmatic proteins by using three different methods in order to prove that this phenotypic technique is a useful taxonomic tool and to provide evidence for subdivision in addition to the biochemical pattern evidence. Protein fingerprinting has been used successfully previously to describe *Lactobacillus pontis* as a new *Lactobacillus* species (50).

Native PAGE was used to differentiate *L. sake* and *L. curvatus*. Furthermore, this method was used to subdivide *L. curvatus* into two clusters which were in agreement with the clusters identified by biochemical differentiation techniques. These *L. curvatus* clusters were differentiated at an *r* level of 0.58, whereas species differentiation was possible at an *r* level of 0.55 (Fig. 1).

The second method which we used was a very sensitive method to analyze the soluble cytoplasmatic protein content and was improved by silver staining. This method was sensitive enough that it could be used for intraspecies differentiation and to identify strain-specific fingerprints. Our results were similar to the results obtained with this technique when it was used to study another closely related *Lactobacillus* species, *L. rhamnosus* (23). Our cluster analysis revealed that *L. sake*

subgroups I and II could be differentiated at an *r* level of 0.81 (Fig. 2 and 3); the *r* level when these subgroups were compared was 0.75. The *r* level when *L. curvatus* subgroup II (cluster C 2) and *L. curvatus* subgroup I (cluster C 1) were compared was 0.81. Subdivision of *L. curvatus* subgroup II was possible only with this method (clusters C 2 and C 3). Because of the uniformity of their biochemical fermentation patterns and the cluster analysis results obtained with the analysis method described below, which included more strains, clusters C 2 and C 3 were identified as *L. curvatus* subgroup II clusters.

SDS-PAGE with colloidal Coomassie blue revealed several clusters, which were clearly related to biochemical patterns in the case of *L. curvatus* and to the proposed subgroups. In case of *L. sake* subdivision was done at an *r* level of 0.61, and the subgroups were related to each other at an *r* level of 0.53 (Fig. 4). The subgroups of *L. curvatus* were confirmed by the results of a protein analysis which clustered the melibiose-fermenting *L. curvatus* strains separately at an *r* level of 0.72 and the other strains at an *r* level of 0.74. The *L. curvatus* subgroups were related to each other at an *r* level of 0.65 (Fig. 5). The usefulness of protein fingerprinting with Coomassie blue staining has been described frequently previously (17, 49, 50).

The status of *L. sake* and *L. curvatus* as separate species was confirmed in this study, and our results are consistent with the results of Dellaglio et al. (6), Beck et al. (1), and Kagermeier-Callaway and Lauer (21). The results of DNA-DNA hybridization and protein fingerprinting analyses did not change the species identifications that were revealed presumptively by biochemical characteristics. In agreement with the subspecies definition of Wayne et al. (51), designation of new *L. sake* and *L. curvatus* subspecies may be possible because strains are genetically closely related but differ phenotypically. Different sources did not influence the subgroups based on phenotypic characteristics. The differences in phenotype were based not only on selected key reactions or utility attributes but also on different fermentation and protein patterns.

Emended description of *Lactobacillus sake*. Cells are rod shaped with rounded ends (0.6 to 0.8 by 2 to 3 μ m) and often slightly curved or irregular, especially during the stationary growth phase, and occur in short chains or singly. Nonmotile. No growth occurs at 45°C, but most strains grow at 42 and 4°C and some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced, often with the L-(+) isomer in excess. The G+C content of the DNA ranges from 42 to 44 mol%. Originally isolated from sake starter and regularly found in fermented meat products, vacuum-packaged meat, sauerkraut, and other fermented plant material.

The species is divided into two subgroups on the basis of protein patterns revealed by SDS-PAGE.

Subgroup I has the following biochemical and physiological features. Acid is produced from fructose, galactose, gluconate, glucose, mannose, melibiose, ribose, and sucrose. Most strains produce acid from arabinose, cellobiose, salicin, and trehalose. A few strains produce acid from lactose and maltose. A few strains fail to produce acid from arabinose. Acid is not produced from amygdalin, inositol, mannitol, melezitose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is always produced from arginine. Most strains grow in the presence of 10% NaCl and produce acetoin from glucose.

Subgroup II differs from subgroup I only in a few biochemical and physiological features. Acid is always produced from salicin and trehalose. A considerable number of strains do not produce acid from arabinose. A few strains produce acid from cellobiose. Acid is not produced from lactose and maltose. Growth occurs in the presence of 10% NaCl. Acetoin is produced from glucose.

The type strain is strain ATCC 15521 (= DSM 20017). The type strain belongs to subgroup I, but its protein patterns are atypical and it differs from other subgroup I strains in some reactions. The type strain produces acid from maltose. Acid is produced weakly from arabinose, gluconate, lactose, salicin, and trehalose. Acid is not produced from cellobiose.

Additional information is given in Table 4.

Emended description of *Lactobacillus curvatus*. Cells are curved, bean-shaped rods with rounded ends (0.7 to 0.9 by 1 to 2 μm) and occur in pairs or short chains; horseshoe-shaped or closed rings are frequently observed. Usually nonmotile. Some strains are motile, but lose motility when they are subcultured. No growth occurs at 45°C, but most strains grow at 42 and 4°C; some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced with the L-(+) isomer often in excess. The G+C content of the DNA ranges from 42 to 44 mol%. Isolated from fermented meat products, vacuum-packaged meat, sauerkraut, and silage.

The species is divided into two subgroups on the basis of protein patterns revealed by SDS-PAGE and native PAGE.

Subgroup I has the following biochemical and physiological features. Acid is produced from fructose, galactose, glucose, maltose, mannose, and ribose. Some strains produce acid from amygdalin, cellobiose, sucrose, and trehalose. A few strains produce acid from lactose and salicin. Acid is not produced from arabinose, gluconate, inositol, mannitol, melezitose, melibiose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is not produced from arginine. Most strains do not grow in the presence of 10% NaCl and do not produce acetoin from glucose.

Subgroup II differs from subgroup I in some biochemical and physiological features. Acid is produced from melibiose, sucrose, and trehalose and is weakly produced from gluconate. Most strains produce acid from salicin. A few strains produce acid from cellobiose. Acid is not produced from amygdalin, lactose, and maltose. No growth occurs in the presence of 10% NaCl. Acetoin is not produced from glucose. The main criterion for differentiation is fermentation of melibiose.

The type strain is strain ATCC 15601 (= DSM 20019). The type strain belongs to subgroup I, but it differs from other subgroup I strains in some reactions. The type strain produces acid from cellobiose, and acid is not produced from amygdalin, sucrose, and trehalose.

Additional information is given in Table 4.

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