Deoxyribonucleic Acid Homology Studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov.

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Deoxyribonucleic acid (DNA)-DNA hybridizations were performed on strains of *Lactobacillus casei*. Our results indicate that this species as presently constituted is genomically very heterogeneous. The majority of strains designated *L. casei* subsp. *casei*, together with members of *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplatanturum*, and *L. casei* subsp. *tolerans*, exhibited high levels of DNA relatedness with each other but were distinct from the type strain of *L. casei* subsp. *casei*. Strains of *L. casei* subsp. *rhamnosus* also formed a genomically homogeneous group unrelated to all members of the other *L. casei* subspecies examined. On the basis of the present and previous findings we suggest that members of *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplatanturum*, and *L. casei* subsp. *tolerans* and the majority of *L. casei* subsp. *casei* strains be given separate species status, for which we propose the names *L. paracasei* sp. nov., *L. paracasei* subsp. *paracasei* (type strain, NCDO 151), and *L. paracasei* subsp. *tolerans* (type strain ATCC 25599). We also propose that *L. casei* subsp. *rhamnosus* be elevated to species status, as *L. rhamnosus* sp. nov. (type strain, ATCC 7469).

Lactobacillus casei is a facultatively heterofermentative species which is found in many habitats (e.g., dairy products, silage, human intestinal tracts, mouths, and sewage). This species is currently poorly defined, and members exhibit considerable phenotypic and genotypic heterogeneity (1, 6). On the basis of phenotypic criteria, five subspecies of *L. casei* are recognized (viz. *L. casei* subsp. *alactosus*, *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplatanturum*, *L. casei* subsp. *rhamnosus*, and *L. casei* subsp. *tolerans*) (7). However, the status and interrelationships of these subspecies are unclear. Johnson (5) found high levels of deoxyribonucleic acid (DNA) relatedness between strains designated *L. casei* subsp. *casei* and *L. casei* subsp. *alactosus*, whereas *L. casei* subsp. *rhamnosus* strains exhibited relatively low levels of homology (<40%) with strains of *L. casei* subsp. *casei*. In a more comprehensive study, Dellaglio et al. (1) found that *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplatanturum*, *L. casei* subsp. *tolerans*, and most strains designated *L. casei* subsp. *casei* formed a single homology group distinct from the type strain of *L. casei* subsp. *casei*. In view of the uncertainty as to the status of these subspecies and the unsatisfactory nomenclature of the *L. casei* group, we examined their genetic interrelationships by using DNA-DNA hybridization.

MATERIALS AND METHODS

Cultures. Details concerning the strains which we used and their sources are given in Table 1.

DNA-DNA hybridization. Cells were grown in MRS broth (2) overnight at 30°C, harvested in the late exponential phase by centrifugation, and washed with distilled water. DNA was prepared by using a modification (3) of the method of Garvie (4). DNA-DNA hybridizations were performed by using the membrane filter method described by Garvie (4), except that we used stringent washing (filters were washed twice in 10 ml of 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and twice in 10 ml of 0.2× SSC).

Biochemical tests. Biochemical tests were performed by

using the API 50CH system (API-Biomerieux) according to the instructions of the manufacturer. Test preparations were incubated at 30°C, and readings were made after 24 and 48 h.

RESULTS AND DISCUSSION

The results of DNA-DNA hybridization experiments are shown in Table 1. Only a single strain (NCDO 173) exhibited a high level of DNA relatedness (80%) with the type strain of *L. casei* subsp. *casei* (NCDO 161). All other *L. casei* strains (including those designated *L. casei* subsp. *alactosus*, *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplatanturum*, *L. casei* subsp. *tolerans*) displayed low levels of relatedness (ca. 10 to 20%) with strain NCDO 161^T (T = type strain). A total of 27 strains labeled *L. casei* subsp. *casei* formed a single homology group when DNA from strain NCDO 151^T was used as the reference. All of the strains of *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplatanturum*, and *L. casei* subsp. *tolerans* examined also displayed high levels of homology (ca. 65 to 85%) with strain NCDO 151^T. Thirteen strains designated *L. casei* subsp. *rhamnosus* formed a relatively homogeneous group which exhibited high levels of relatedness (65 to 100%) with *L. casei* subsp. *rhamnosus* NCDO 243^T. The *L. casei* subsp. *rhamnosus* strains showed only low levels of relatedness (ca. 10 to 20%) to members of the strain NCDO 161^T and NCDO 151^T homology groups.

The results of this present study clearly show that the species *L. casei* as currently recognized is genomically heterogeneous, in accordance with the earlier findings of Dellaglio et al. (1). The DNA hybridization data indicate that most members of the species (including strains of *L. casei* subsp. *alactosus*, *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplatanturum*, *L. casei* subsp. *rhamnosus*, and *L. casei* subsp. *tolerans*) are genetically unrelated to the type strain of *L. casei* subsp. *casei* (strain NCDO 161). The recovery of the majority of strains designated *L. casei* subsp. *casei* together with members of *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplatanturum*, and *L. casei* subsp. *tolerans* in a single homology group displaying low levels of homology with the type strain indicates that these strains warrant a

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TABLE 1. Levels of DNA homology (under stringent conditions) for strains of *L. casei*, *L. paracasei*, *L. rhamnosus*, and other *Lactobacillus* species

Strain	Source	% Relative binding with [³ H]DNA from:		
		<i>L. casei</i> NCDO 161 ^T	<i>L. paracasei</i> NCDO 151 ^T	<i>L. rhamnosus</i> NCDO 243 ^T
<i>L. casei</i>				
NCDO 161 ^T	F. J. Orland, ATCC 393 ^T ; DSM 20011 ^T	100	16	19
NCDO 173	R. P. Tittsler, C27	80	ND ^a	13
<i>L. paracasei</i>				
NCDO 151 ^T	Received as <i>L. casei</i>	15	100	13
NCDO 205	M. E. Sharpe; <i>L. casei</i> subsp. <i>alactosus</i>	ND	71	12
NCDO 206	M. E. Sharpe; <i>L. casei</i> subsp. <i>alactosus</i>	ND	63	ND
NCDO 242	M. E. Sharpe	ND	73	ND
NCDO 327	J. W. N. McIntosh; from dental caries; ATCC 11974, NCIB 1407	15	74	12
NCDO 348	J. G. Davis; CC72; NCIB 3254	10	61	ND
NCDO 680	R. G. Jensen via M. E. Sharpe, ATCC 11582; oral source	ND	62	10
NCDO 1202	M. E. Sharpe; cheese starter	16	62	12
NCDO 1857	J. Elliot; from cheddar cheese	15	77	ND
NCDO 1974	O. Kandler; from pasteurized milk; <i>L. casei</i> subsp. <i>tolerans</i> DSM 20012	15	81	13
NCDO 1977	O. Kandler; from pasteurized milk; <i>L. casei</i> subsp. <i>pseudoplatantarum</i> DSM 20207	ND	71	ND
NCDO 2713	M. Rogosa; from human saliva; <i>L. casei</i> subsp. <i>alactosus</i> ; ATCC 27216, DSM 20020	26	85	12
NCDO 2743	Abo-Elnaga; from milking machine; <i>L. casei</i> subsp. <i>pseudoplatantarum</i> ; ATCC 25598, DSM 20008	13	65	11
NCIB 9709 ^T	<i>L. casei</i> subsp. <i>tolerans</i>	15	86	14
C1	M. E. Sharpe	ND	68	ND
C2	M. E. Sharpe	ND	70	ND
C4	M. R. Whitehead; from New Zealand cheese	ND	69	ND
C8	M. E. Sharpe	ND	68	13
C11	W. S. Graves	ND	75	ND
C13	M. E. Sharpe	ND	66	ND
C19	A. H. Pederson; dairy source	12	67	ND
C22	J. M. Sherman; dairy source	13	67	ND
C23	M. R. Whitehead; dairy source	ND	63	ND
C35	M. E. Sharpe; Yugoslavian sour milk	ND	68	ND
C40	M. E. Sharpe	19	61	ND
C41	M. E. Sharpe	ND	69	ND
C42	From dairy source	11	65	ND
C43	R. P. Tittsler; dairy source	ND	69	ND
C44	From sour milk	ND	61	ND
C45	From sour milk	ND	61	ND
C46	From cheese	11	72	ND
C47	M. E. Sharpe	11	86	ND
A22/73	From human blood (PHLS) ^b	ND	61	ND
A112/73	From lung abscess (PHLS)	ND	65	ND
<i>L. rhamnosus</i>				
NCDO 243 ^T	ATCC 7469 ^T , DSM 20021 ^T , CCM 1825 ^T	15	9	100
NCDO 86	M. E. Sharpe	ND	ND	80
NCDO 244	E. Davis; ATCC 11981, NCIB 6557	ND	6	65
NCDO 252	M. E. Sharpe; ATCC 11982	ND	ND	100
NCDO 330	F. J. Orland	ND	ND	100
NCDO 1051	A. C. Hayward; from white Cheshire cheese	ND	10	80
NCDO 1657	Mutant derived from ATCC 7469	ND	ND	68
NCDO 1856	ATCC 11443	ND	ND	100
NCDO 1858	J. Elliot	ND	ND	79
NCTC 10302	M. E. Sharpe; from human saliva	ND	ND	65
C10	M. E. Sharpe; dairy source	ND	ND	80
A17/70	From lymph node (PHLS)	ND	ND	90
A103/70	Clinical source (PHLS)	ND	ND	75
100171	From endocarditis (PHLS)	ND	ND	69
GK1	Clinical source (Hillingdon Hospital, Uxbridge, United Kingdom)	ND	ND	75
<i>Lactobacillus agilis</i>				
NCDO 2744 ^T	From municipal sewage; DSM 20509 ^T , NCIB 11716 ^T	4	5	5

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TABLE 1—Continued

Strain	Source	% Relative binding with [³ H]DNA from:		
		<i>L. casei</i> NCDO 161 ^T	<i>L. paracasei</i> NCDO 151 ^T	<i>L. rhamnosus</i> NCDO 243 ^T
<i>Lactobacillus alimentarius</i> NCDO 2329 ^T	From marinated fish product; DSM 20249 ^T , ATCC 29643 ^T	4	4	5
<i>Lactobacillus bavaricus</i> NCDO 2588 ^T	From sauerkraut; DSM 20269 ^T	4	9	5
<i>Lactobacillus brevis</i> NCDO 1749 ^T	ATCC 14869 ^T , DSM 20054 ^T	3	5	6
<i>L. curvatus</i> NCDO 2739 ^T	From milk; DSM 20019 ^T	8	7	5
NCDO 1039	A. C. Hayward; from Italian hard cheese	ND	ND	4
<i>Lactobacillus helveticus</i> NCDO 102	M. E. Sharpe	3	3	4
" <i>Lactobacillus murinus</i> " NCDO 2175	M. E. Sharpe	4	4	6
<i>Lactobacillus plantarum</i> NCDO 1752 ^T	From pickled cabbage; ATCC 14917 ^T , DSM 20174 ^T	4	5	5

^a ND, Not determined.^b PHLS, Public Health Laboratory Service, Colindale, United Kingdom

new species, for which we propose the name *Lactobacillus paracasei* (pa.ra.ca'se.i. Gr.prep. *para*, resembling; L.gen.n. *casei*, a specific epithet; M.L.adj. *paracasei*, resembling *L. casei*). We believe that strains previously designated *L. casei* subsp. *tolerans* are phenotypically so distinct that they should retain separate subspecific status (i.e., *L. paracasei* subsp. *tolerans*).

The high levels of homology exhibited by members of *L. casei* subsp. *rhamnosus* and the low levels of relatedness to all of the other strains examined indicate that this taxon should also be elevated to species rank, as first suggested by Dellaglio and associates (1). We propose the name *Lactobacillus rhamnosus* (rham.no'sus. M.L.adj. *rhamnosus*, pertaining to rhamnose) sp.nov. is proposed for this organism. Full descriptions of *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp. *tolerans*, and *L. rhamnosus* are given below. These taxa can be distinguished from each other and from *L. casei* by using the criteria shown in Table 2.

Description of *Lactobacillus paracasei* subsp. *paracasei*. Cells are rod shaped (ca. 0.8 to 1.0 by 2.0 to 4.0 µm), often with square ends, and occur singly or in chains. Nonmotile. Grows at 10 and 40°C; some strains grow at 5 and 45°C. Facultatively heterofermentative. L-(+)-Lactic acid is produced; a few strains produce inactive lactic acid due to the activity of L-lactic acid racemase. Acid is produced from amygdalin, arbutin, cellobiose, D-fructose, galactose, D-glucose, maltose, mannitol, and D-mannose, melezitose, N-acetylglucosamine, salicin, D-tagatose, and trehalose. Most strains produce acid from β-gentiobiose, gluconate, lactose, α-methyl-D-glucoside, ribose, sorbitol, sucrose, and D-turanose. A few strains produce acid from adonitol, D-arabinose, D-arabitol, dulcitol, D-fucose, glycerol, inositol, inulin, D-lyxose, ribitol, L-sorbose, and starch. Acid is not produced from L-arabinose, L-arabitol, erythritol, L-fucose, 2-keto-gluconate, 5-keto-gluconate, α-methyl-mannoside, β-methyl-xyloside, rhamnose, xylitol, D-xylose, and L-xylose. Ammonia is not produced from arginine. Esculin is hydrolyzed. Urease negative. Murein type Lys-D-Asp. The guanine-plus-cytosine content of the DNA ranges from 45 to 47 mol%. Isolated from dairy products, sewage, silage, humans, and clinical sources.

The type strain is strain NCDO 151. In most respects the description of the type strain resembles the description of

TABLE 2. Differential characteristics of *L. casei*, *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp. *tolerans*, and *L. rhamnosus*^a

Acid produced from:	<i>L. casei</i> (2 strains)	<i>L. paracasei</i> subsp. <i>paracasei</i> (30 strains)	<i>L. paracasei</i> subsp. <i>tolerans</i> (2 strains)	<i>L. rhamnosus</i> (10 strains)
Adonitol	— ^b	6/30 ^c	—	—
Amygdalin	+	+	—	+
D-Arabinose	—	2/30	—	1/10
Arbutin	+	+	—	+
Cellobiose	+	+	—	+
Dulcitol	—	7/30	—	3/10
Esculin	+	+	—	+
D-Fucose	—	3/30(w) ^d	—	—
L-Fucose	1/2	—	—	1/10
β-Gentiobiose	—	27/30	—	+
Gluconate	+	28/30	+(w)	+
Glycerol	—	3/30(w)	—	6/10(w)
Inositol	—	9/30(w)	—	9/10(w)
Inulin	—	9/30	—	—
Lactose	—	23/30	+	+
D-Lyxose	—	7/30(w)	—	6/10(w)
Maltose	1/2	+	—	+
Mannitol	+	+	—	+
α-Methyl-glucoside	—	16/30(w)	—	9/10
Melezitose	+	+	—	+
Rhamnose	—	—	—	+
Ribose	—	29/30	—	+
Salicin	+	+	—	+
Sorbitol	—	19/30	—	+
L-Sorbose	—	10/30	—	8/10
Starch	—	2/30	—	—
Sucrose	—	29/30(w)	—	+(w)
Trehalose	+	+	—	+
D-Turanose	—	28/30	—	9/10

^a All strains produce acid from N-acetyl-glucosamine, D-fructose, galactose, D-glucose, D-mannose, and D-tagatose; all fail to produce acid from L-arabinose, D-arabitol, L-arabitol, erythritol, 2-keto-gluconate, 5-keto-gluconate, α-methyl-mannoside, β-methyl-xyloside, xylitol, D-xylose, and L-xylose.

^b +, All strains positive; —, all strains negative.

^c Number of strains positive/number of strains tested.

^d (w), Some reactions are weak.

the subspecies. The type strain does not grow at 45°C. The type strain produces acid from β -gentiobiose, gluconate, lactose, and sucrose; acid is not produced from D-arabinose, adonitol, dulcitol, glycerol, inositol, inulin, D-lyxose, α -methyl-D-glucoside, sorbitol, and L-sorbose.

Description of *Lactobacillus paracasei* subsp. *tolerans*. Cells are rod shaped (ca. 0.8 to 1.0 by 2.0 to 4.0 μ m), often with square ends, and occur singly or in chains. Nonmotile. Grows at 10 and 37°C; no growth at 40°C. Survives heating at 72°C for 40 s. Facultatively heterofermentative. L-(+)-Lactic acid is produced. Acid is produced from D-fructose, galactose, D-glucose, lactose, D-mannose, N-acetylglucosamine, and D-tagatose. Acid is not produced from adonitol, amygdalin, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D-fucose, L-fucose, β -gentiobiose, glycerol, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, D-lyxose, maltose, mannitol, α -methyl-D-glucoside, α -methyl-mannoside, β -methyl-xyloside, melezitose, rhamnose, ribose, salicin, sorbitol, L-sorbose, starch, sucrose, trehalose, D-turanose, xylitol, D-xylose, and L-xylose. Ammonia is not produced from arginine. Esculin is not hydrolyzed. Urease negative. Murein type Lys-D-Asp. The guanine-plus-cytosine content of the DNA is 45 to 47 mol%. Isolated from dairy products. The type strain is strain ATCC 25599 (= NCIB 9709 = NCFB 2774). The description of the type strain corresponds to that of the subspecies.

Description of *Lactobacillus rhamnosus* sp. nov. Cells are rod shaped (ca. 0.8 to 1.0 by 2.0 to 4.0 μ m), often with square ends, and occur singly or in chains. Nonmotile. Grows at 15 and 45°C; most strains grow at 10°C, and some strains grow at 48°C. Facultatively heterofermentative. L-(+)-Lactic acid is produced. Acid is produced from amygdalin, arbutin, cellobiose, D-fructose, galactose, β -gentiobiose, gluconate, D-glucose, lactose, mannitol, D-mannose, maltose, melezitose, N-acetylglucosamine, rhamnose, ribose, salicin, sorbitol, D-tagatose, and trehalose. Most strains produce acid from glycerol, inositol, D-lyxose, α -methyl-

D-glucoside, L-sorbose, sucrose, and D-turanose. Acid is not produced from adonitol, L-arabinose, D-arabitol, L-arabitol, erythritol, D-fucose, inulin, 2-keto-gluconate, 5-keto-gluconate, α -methyl-mannoside, β -methyl-xyloside, starch, xylitol, D-xylose, and L-xylose. Ammonia is not produced from arginine. Esculin is hydrolyzed. Urease negative. Murein type Lys-D-Asp. The guanine-plus-cytosine content of the DNA ranges from 45 to 47 mol%. Isolated from dairy products, sewage, humans, and clinical sources.

The type strain is strain ATCC 7469 (= NCDO 243). In most respects the description of the type strain resembles the description of the species. The type strain produces acid from inositol, D-lyxose, α -methyl-D-glucoside, L-sorbose, and D-turanose; acid is not produced from D-arabinose, dulcitol, and glycerol.

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