Comparative Sequence Analyses of the Genes Coding for 16S rRNA of *Lactobacillus casei*-Related Taxa

KATSUMI MORI,^{1*} KOJI YAMAZAKI,² TOMOHARU ISHIYAMA,^{1†} MASAHIRO KATSUMATA,^{1‡}
KAYO KOBAYASHI,^{1§} YUJI KAWAI,² NORIO INOUE,² AND HARUO SHINANO²

Systematic Microbiology Laboratory, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries,
2-1-2 Kannondai, Tsukuba, Ibaraki 305,¹ and Faculty of Fisheries, Hokkaido University,
Hakodate, Hokkaido 041,² Japan

The primary structures of the 16S rRNA genes of the type strains of *Lactobacillus casei* and related taxa were determined by PCR DNA-sequencing methods. The sequences of *Lactobacillus casei*, *Lactobacillus zeae*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* were different. The K_{nuc} values ranged from 0.0040 to 0.0126. On the basis of the K_{nuc} values and the levels of DNA-DNA relatedness among the strains of these species, the *L. casei*-related taxa should be classified in the following three species: *L. zeae*, which includes the type strains of *L. zeae* and *L. casei*; a species that includes the strains of *L. paracasei* and *L. casei* ATCC 334; and *L. rhamnosus*.

Members of *Lactobacillus casei* are gram-positive, facultatively anaerobic, catalase-negative, facultatively heterofermentative, non-spore-forming rods and are isolated from many habitats (e.g., meats, milk, dairy products, sour dough, silage, and sewage). Five subspecies of this species, *L. casei* subsp. *casei* (Orla-Jensen 1916) Hansen and Lessel 1971, *L. casei* subsp. *alactosus* Mills and Lessel 1973, *L. casei* subsp. *pseudopiantarum* Abo-Elnaga and Kandler 1965, *L. casei* subsp. *rhamnosus* Hansen 1968, and *L. casei* subsp. *tolerans* Abo-Elnaga and Kandler 1965, were recognized on the Approved Lists of Bacterial Names on the basis of phenotypic criteria (16). Then, in *Bergey's Manual of Systematic Bacteriology* (11), the five subspecies of *L. casei* were reclassified into four subspecies (*L. casei* subsp. *alactosus* was combined with *L. casei* subsp. *casei*) on the basis of the comprehensive studies of Dellaglio et al. (7). In 1989, Collins et al. (3) reclassified the subspecies of *L. casei* into the following three species on the basis of DNA-DNA relatedness data: *L. casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*. In 1991, Dellaglio et al. requested a change in the designation of the neotype strain of *L. casei* subsp. *casei* and rejection of the name *L. paracasei* as a synonym of *L. casei* (8). However, the Judicial Commission of the International Committee on Systematic Bacteriology voted against this request (20). In 1996, Dicks et al. proposed that the species name *Lactobacillus zeae* should be revised and that strain ATCC 334 should be designated the neotype strain of *L. casei* subsp. *casei* and thus the name *L. paracasei* should be rejected (9).

The study of 16S rRNA sequences is now considered to be a powerful technique for determining the phylogenetic relationships of microorganisms (1, 4–6, 13, 19, 22, 23). Weisburg et al. determined the 16S rRNA sequence of the type strain of *L. casei* (accession number M23928), Woese et al. determined the 16S rRNA sequence of the type strain of *L. rhamnosus* (accession number M58815), and Hammes et al. described the phylogenetic relationship between *L. casei* and *L. rhamnosus*

(10). Collins also determined the 16S rRNA sequences of the type strains of *L. casei*, *L. paracasei*, and *L. rhamnosus* (6).

However, the 16S rRNA sequences of all of the type strains of the *L. casei* subspecies and the phylogenetic relationships among these strains have not been analyzed, so the classification of the *L. casei* group is not yet stable. Therefore, we analyzed ca. 1,520 nucleotides of the genes coding for the 16S rRNA (16S rDNA) in all of the type strains of the *L. casei* subspecies and closely related strains and then determined the phylogenetic relationships of these organisms.

MATERIALS AND METHODS

Bacterial strains and cultivation. The strains used in this study are shown in Table 1. These strains were grown aerobically on Briggs agar (Nissui Seiyaku, Inc., Tokyo, Japan) slants for 2 to 3 days at 30°C.

Extraction and determination of the 16S rDNA sequence. Total DNA was extracted and purified from a loopful of cells of each strain with an InstaGene purification matrix (Bio-Rad Laboratories, Richmond, Calif.) according to the protocol for bacteria (2). The 16S rDNA fragments in the total DNA were amplified by the modified PCR methods of Wittwer and Garling (21) by using a model 1605 air thermocycler (Idaho Technology, Inc.) and 50- μ l glass capillary tubes. Eleven fragments of the 16S rDNA were amplified to facilitate amplification and sequencing. The 11 pairs of primers used for amplification are shown in Table 2. The sequence of primer –21M13 was connected to the 5' end region of one primer from each pair, and so a strand of the PCR product contained a complementary sequence of –21M13 in the 3' end region to which the –21M13 universal fluorescent primer annealed for sequencing. The nucleotide sequences of the strands which contained the complementary sequence of –21M13 were determined with a model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) by the dideoxynucleotide chain termination method modified for use with the –21M13 universal fluorescent primer and a *Taq* dye primer cycle sequencing kit (Applied Biosystems, Inc.) (14).

Data analysis. The sequences that were generated were aligned by using the program DNASIS (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). Homology values and evolutionary distances (K_{nuc} values) (12, 18) were calculated, and an unrooted phylogenetic tree was produced by the neighbor-joining method (15), using Saitou's NJ computer program.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rDNAs determined in this study have been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

Sequences of the 16S rDNAs of *L. casei*-related strains. The sequences of more than 1,520 nucleotides of the 16S rDNAs (homologous to positions 28 to 1,521 of the *Escherichia coli* 16S rRNA sequence) were determined for all of the strains tested by the PCR DNA-sequencing method. Weisburg et al.

* Corresponding author.

† Present address: Ishiyama Miso-Shoyu Co., Ltd., Niigata-shi, Japan.

‡ Present address: Hagoromo Foods Co., Ltd., Shimizu-shi, Japan.

§ Present address: Du Pont K.K., Meguro-ku, Tokyo, Japan.

TABLE 1. Strains tested, accession numbers of 16S rDNAs, and DNA-DNA relatedness among the strains

Strain	% DNA relatedness with DNA from:				Basonym (accession no. of 16S rDNA)
	ATCC 393 ^{Ta}	NCDO 161 ^{Tb}	ATCC 334 ^a	NCDO 151 ^{Tb}	
<i>L. zeae</i> ATCC 15820 ^T (= DSM 20178 ^T = NCIB 9537 ^T)	80		42		<i>L. rhamnosus</i> ATCC 15820 (D86516)
<i>L. casei</i> JCM 1134 ^T (= ATCC 393 ^T = NCDO 161 ^T)	100	100	36	16	<i>L. casei</i> subsp. <i>casei</i> JCM 1134 ^T (D16551)
<i>L. casei</i> JCM 8129 (= NCDO 173)		80			<i>L. casei</i> subsp. <i>casei</i> JCM 8129 (D86518)
<i>L. casei</i> ATCC 334	43		100		<i>L. casei</i> subsp. <i>casei</i> ATCC 334 (D86517)
<i>L. paracasei</i> subsp. <i>paracasei</i> JCM 8130 ^T (= NCDO 151 ^T)	22	15	95	100	<i>L. casei</i> subsp. <i>casei</i> JCM 8130 (D79212)
<i>L. paracasei</i> subsp. <i>paracasei</i> JCM 1133 (= ATCC 27216 = NCDO 2713)	50	26	85	85	<i>L. casei</i> subsp. <i>alactosus</i> JCM 1133 ^T (D16548)
<i>L. paracasei</i> subsp. <i>paracasei</i> JCM 1181 (= NCDO 2743 = DSM 20008)	33	13	86	65	<i>L. casei</i> subsp. <i>pseudoplantarum</i> JCM 1181 ^T (D16549)
<i>L. paracasei</i> subsp. <i>tolerans</i> JCM 1171 ^T (= ATCC 25599 ^T = DSM 20258 ^T)		15		86	<i>L. casei</i> subsp. <i>tolerans</i> JCM 1171 ^T (D16550)
<i>L. rhamnosus</i> JCM 1136 ^T (= ATCC 7469 ^T = NCDO 243 ^T)	45	15	47	9	<i>L. casei</i> subsp. <i>rhamnosus</i> JCM 1136 ^T (D16552)

^a Data from references 8 and 9.^b Data from reference 3.

reported the 16S rRNA sequence of type strain ATCC 393 of *L. casei* (accession number M23928). In the present study, the sequence of type strain JCM 1134 of *L. casei* (accession number D16551) included 23 bases that Weisburg et al. reported as unknown. One base determined in this study did not agree with

the data of Weisburg et al. The nucleotide at position 534 (*E. coli* numbering) was determined to be T in our study and C in the study of Weisburg et al. Woese et al. determined the 16S rRNA sequence of type strain DSM 20021 of *L. rhamnosus* (accession number M58815). Our sequence data for type strain

TABLE 2. Sequences of oligonucleotides used as primers for 16S rDNA amplification

Pair no.	Primer	Sequence(5'-3') ^a
	-21M13	TGTAACACGACGGCCAGT
11	M27F 520R	(-21M13)-AGAGTTTGATCCTGGCTCAG ACCGCGGC(G/T)GCTGGC
12	M357F 777R	(-21M13)-CTACGGGAGGCAGCAG GTATCTAATCCTGTT(T/C)GCT
13	M703F 1080R	(-21M13)-GCGGTGAAATGCGTAG CCCAACATCTCACGAC
14	M920F 1400R	(-21M13)-AAACTCAAAGGAATTGACGG ACGGGCGGTGTGT(G/A)C
15	M1240F 1522R	(-21M13)-ACACACGTGCTACAAT AAGGAGGTGATCCA(A/G)CCGCA
161	M246R N27F	(-21M13)-TTACC(T/C)CACC(A/T)ACTAGCT AGAGTTTGATCCTGGCTCAG
162	M400R N27F	(-21M13)-AAAICCTTC(T/A)TCAC(T/A)CAC AGAGTTTGATCCTGGCTCAG
171	M700R 359F	(-21M13)-AT(A/C)TCTACGCATT(T/C)CACCGC CTACGGGAGGCAGCAGTAG
18	M920R 530F	(-21M13)-CCGTCAATTC(C/A)TTT(G/A)AGTTT GTGCCAGCCGCCGCGG
19	M1240R 790F	(-21M13)-CCATTGTAGCACGTGTGTAG AGC(A/G)AACAGGATTAGATAC
20	M1522R 1080F	(-21M13)-AAGGAGGTGATCCA(A/G)CCGCA GT(C/T)GTGA(G/A)ATGTTGGGTAAAG

^a (-21M13), sequence of -21M13.

FIG. 1. Secondary structures of 16S rRNAs of the type strains of *L. casei* and related taxa at *E. coli* 16S rRNA positions 66 to 103. R, A or G; W, A or T.

JCM 1136 of *L. rhamnosus* (accession number D16552) included 47 bases that Woese et al. reported as unknown. Also, only one base in our sequence did not agree with the data of Woese et al.; the nucleotide at position 1028 (*E. coli* numbering) in our sequence was C, and there was a gap at this position in the sequence of Woese et al. Collins et al. reported that they determined ca. 1,490 bases of the 16S rRNA sequences of *L. casei* NCDO 161^T (T = type strain), *L. paracasei* NCDO 151^T, and *L. rhamnosus* NCDO 243^T and that more than 99% sequence homology was exhibited by these three species (6). The data for *L. casei* only (accession number X61135) appeared in the nucleotide sequence databases. Although we also used type strain JCM 1134 of *L. casei*, our sequence data

TABLE 3. K_{nuc} values for 16S rDNAs of *L. casei*-related species

Species	K_{nuc} value with:		
	<i>L. casei</i>	<i>L. zeae</i>	<i>L. paracasei</i>
<i>L. zeae</i>	0.0040		
<i>L. paracasei</i>	0.0073	0.0086	
<i>L. rhamnosus</i>	0.0086	0.0126	0.0113

FIG. 2. Unrooted tree showing the phylogenetic relationships of *L. casei* and related taxa. The evolutionary distance between any two taxa is the sum of the lengths of the horizontal lines between them.

(accession number D16551) did not agree with the data of Collins et al. (accession number X61135) at 31 positions. At these 31 positions, our data agreed completely with the data of Weisburg et al. (accession number M23928). Our data included 125 bases that Collins et al. reported as unknown.

In a comparison of the sequences of all of the strains tested, we found that the sequences of the strains belonging to the species *L. paracasei* (that is, *L. paracasei* subsp. *paracasei* JCM 8130^T [basonym, *L. casei* subsp. *casei* JCM 8130], *L. paracasei* subsp. *paracasei* JCM 1133 [basonym, *L. casei* subsp. *alactosus* JCM 1133^T], *L. paracasei* subsp. *paracasei* JCM 1181 [basonym, *L. casei* subsp. *pseudoplatanturum* JCM 1181^T], and *L. paracasei* subsp. *tolerans* JCM 1171^T [basonym, *L. casei* subsp. *tolerans* JCM 1171^T]) and the sequence of *L. casei* ATCC 334 were the same. The sequences of the *L. casei* strains (*L. casei* JCM 1134^T and *L. casei* JCM 8129) were also the same. However, the sequences of the type strains of the four species studied (*L. casei* JCM 1134, *L. zeae* ATCC 15820, *L. paracasei* JCM 8130, and *L. rhamnosus* JCM 1136) were different. The number of base differences between *L. casei* and *L. zeae* was 6, and the numbers of base differences among the other species were 11 to 19.

Comparative analysis of the potential secondary structures of the 16S rRNAs estimated from the 16S rDNA sequences of the *L. casei*-related strains revealed a region in which there are differences. This is the region from position 69 to position 100 (*E. coli* numbering); the potential secondary structures are shown in Fig. 1. At present it is still not known whether the function of 16S rRNA is influenced by such differences in the secondary structure.

TABLE 4. Sequence signatures in the 16S rDNAs of *L. casei*-related taxa

Species	Nucleotide at position ^a :											
	73	75	76	84	85	96	97	98	104	107	108	110
<i>L. casei</i>	T	G	G	A	C	C	T	G	C	C	T	A
<i>L. zeae</i>	T	G	G	A	C	T	C	G	C	C	T	A
<i>L. paracasei</i>	C	C	G	T	C	C	C	G	C	C	A	G
<i>L. rhamnosus</i>	C	G	A	A	A	T	C	T	T	T	T	G

^a Position from the 5' end of the 16S rRNA sequence of *L. casei*.

Phylogenetic relationships among *L. casei*, *L. zeae*, *L. paracasei*, and *L. rhamnosus*. Evolutionary distances (K_{nuc} values) among the strains tested were calculated (Table 3), and a phylogenetic tree based on these K_{nuc} values is shown in Fig. 2. The evolutionary distance between *L. casei* and *L. rhamnosus* agreed with the distance reported by Hammes et al. (10).

For species definitions, sequence analysis of 16S rRNA is the level at which DNA pairing studies need to be performed (17). The K_{nuc} value between *L. casei* JCM 1134^T (or *L. casei* JCM 8129) and *L. zeae* ATCC 15820^T was 0.0040, about one-half of the values determined for the other species. Because of this low K_{nuc} value and the high DNA-DNA relatedness (Table 1) between these strains reported by Dellaglio et al. (8), it was thought that these organisms should be classified in the species *L. zeae*. The sequences of the strains of *L. paracasei* and *L. casei* ATCC 334 (which Dicks et al. has proposed to designate the neotype strain of *L. casei* subsp. *casei*) were the same, and Dellaglio et al. also reported high DNA-DNA relatedness (Table 1) between *L. paracasei* NCDO 151^T and *L. casei* ATCC 334 (8). Therefore, the strains of *L. paracasei* and *L. casei* ATCC 334 should be classified in one species. The sequence of *L. rhamnosus* JCM 1136^T was clearly distinct from the sequences of the other species tested and was consistent with the classification of *L. rhamnosus* proposed by Collins et al. (3).

Sequence signature. The sequence alignments demonstrated that *L. casei*, *L. zeae*, *L. paracasei*, and *L. rhamnosus* could be readily distinguished from each other. The sequence signatures which distinguish these four species from each other are shown in Table 4.

ACKNOWLEDGMENTS

We are grateful to H. Oyaizu, The University of Tokyo, for technical advice and for supplying primers 520R, 1080R, and 1400R.

This study was supported in part by a grant-in-aid (Silage Program) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

REFERENCES

- Bentley, R. W., J. A. Leigh, and M. D. Collins. 1991. Intra-genetic structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. *Int. J. Syst. Bacteriol.* **41**:487-494.
- Bio-Rad Laboratories. 1992. InstaGene purification matrix manual. Bio-Rad Laboratories, Richmond, Calif.
- Collins, M. D., B. A. Phillips, and P. Zanoni. 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. *Int. J. Syst. Bacteriol.* **39**:105-108.
- Collins, M. D., C. Ash, J. A. E. Farrow, S. Wallbanks, and A. M. Williams. 1989. 16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa, description of *Vagococcus fluvialis* gen. nov., sp. nov. *J. Appl. Bacteriol.* **67**:453-460.
- Collins, M. D., A. M. Williams, and S. Wallbanks. 1990. The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. *FEMS Microbiol. Lett.* **70**:255-262.
- Collins, M. D., U. Rodrigues, C. Ash, M. Aguirre, J. A. E. Farrow, A. Martinez-Murcia, B. A. Phillips, A. M. Williams, and S. Wallbanks. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol. Lett.* **77**:5-12.
- Dellaglio, F., V. Bottazzi, and M. Vescovo. 1975. Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium* Orla-Jensen. *Int. J. Syst. Bacteriol.* **25**:160-172.
- Dellaglio, F., L. M. T. Dicks, M. Du Toit, and S. Torriani. 1991. Designation of ATCC 334 in place of ATCC 393 (NCDO 161) as the neotype strain of *Lactobacillus casei* subsp. *casei* and rejection of the name *Lactobacillus paracasei* (Collins et al., 1989). Request for an opinion. *Int. J. Syst. Bacteriol.* **41**:340-342.
- Dicks, L. M. T., E. M. Du Plessis, F. Dellaglio, and E. Lauer. 1996. Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. *Int. J. Syst. Bacteriol.* **46**:337-340.
- Hammes, W. P., N. Weiss, and W. Holzapfel. 1992. The genera *Lactobacillus* and *Carnobacterium*, p. 1535-1594. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed., vol. 2. Springer-Verlag, New York, N.Y.
- Kandler, O., and N. Weiss. 1986. Genus *Lactobacillus* Beijerinck 1901, p. 1209-1234. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
- Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**:111-120.
- Martinez-Murcia, A. J., and M. D. Collins. 1990. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol. Lett.* **70**:73-84.
- Perkin-Elmer Co. 1993. 373 DNA sequencing system user's manual. Perkin-Elmer Co., Norwalk, Conn.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.
- Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **30**:225-420.
- Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846-849.
- Tamura, K., and M. Nei. 1993. Estimation of the numbers of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**:512-526.
- Walbank, S., A. T. Martinez-Murcia, J. L. Fryer, B. A. Phillips, and M. D. Collins. 1990. 16S rRNA determination for members of genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. *Int. J. Syst. Bacteriol.* **40**:224-230.
- Wayne, L. G. 1994. Action of the Judicial Commission of the International Committee on Systematic Bacteriology on requests for opinion published between January 1985 and July 1993. *Int. J. Syst. Bacteriol.* **44**:177-178.
- Wittwer, C. T., and D. J. Garling. 1991. Rapid cycle DNA amplification: time and temperature optimization. *BioTechniques* **10**:76-83.
- Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221-271.
- Yang, D., and C. R. Woese. 1989. Phylogenetic structure of the "*Leuconostoc*": an interesting case of a rapidly evolving organism. *Syst. Appl. Microbiol.* **12**:154-149.