

Genomic characterization reconfirms the taxonomic status of *Lactobacillus parakefiri*

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Whole-genome sequencing was performed for *Lactobacillus parakefiri* JCM 8573^T to confirm its hitherto controversial taxonomic position. Here, we report its first reliable reference genome. Genome-wide metrics, such as average nucleotide identity and digital DNA-DNA hybridization, and phylogenomic analysis based on multiple genes supported its taxonomic status as a distinct species in the genus *Lactobacillus*. The availability of a reliable genome sequence will aid future investigations on the industrial applications of *L. parakefiri* in functional foods such as kefir grains.

Key words: *Lactobacillus parakefiri*, taxonomy, lactic acid bacteria, whole-genome sequence

Type strains hold significant positions in bacterial nomenclature: the taxonomic affiliation of any other isolate is identified on the basis of comparison with the type strains. For example, an isolate showing a DNA-DNA hybridization (DDH) similarity of more than 70% to the type strain of a certain species is considered to belong to the same species. The DDH value of 70%, a classical threshold described nearly 30 years ago, still remains for the gold standard for species boundary, although it has been partly supplemented by the use of 16S rRNA gene sequence similarity [1]. However, recently, the use of genome-wide metrics, such as average nucleotide identity (ANI) and digital DDH (dDDH), has been proposed as a replacement for the laborious process of DDH. ANI is calculated from the mean identity of homologous regions between two genomes, and the cutoff value of 95% is widely acknowledged as the threshold to delineate two species [2, 3]. The dDDH value is also based on the sequence alignment between a

given pair of genomes, as implemented by the Genome-to-Genome Distance Calculator (GGDC, <http://ggdc.dsmz.de>), which infers an *in-silico* analogue of DDH with confidence intervals [4]. These sequence-based methods are reproducible and scalable and thus may be applied in the detection and validation of taxonomic mislabeling in public sequence databases [5–7]. In our previous study, we used ANI to assess the taxonomic status of 718 publicly available genomes of the genus *Lactobacillus*, the largest group of lactic acid bacteria, with over 180 known species [8]. We found mislabeling of organism names in several genomes, possibly caused by taxonomic misidentification or human errors such as sample mix-ups during experimental procedures or data handling. Moreover, our results suggested that even genomes for type strains might be erroneous in several species, including *L. parakefiri* and *L. homohiochii*.

L. parakefiri is a heterofermentative lactic acid bacterium described by Takizawa *et al.* in 1994, with strain GCL 1731^T (= JCM 8573^T = DSM 10551^T = ATCC 51648^T) designated as its type strain [9]. It was originally isolated from kefir grains and, together with another kefir-isolated species, *L. kefiri*, belongs to the *L. buchneri* group [10, 11]. The complex compositions and mechanisms of kefir grain microbiota have been studied extensively because of the health benefits associated with kefir ingestion [12, 13]. Recently, conflicting views were reported by two research groups for *L.*

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parakefiri on the basis of genomic analyses, resulting in controversies about the taxonomic status of *L. parakefiri*. Zheng *et al.* suggested that *L. parakefiri* is a later heterotypic synonym of *L. kefiri* [14], whereas Sun *et al.* acknowledged it as the species with the largest genome in the genus *Lactobacillus* [15]. In both studies, the draft genome was reconstructed independently using the raw sequencing reads for DSM 10551^T deposited under the accession numbers SRR1151226 and ERR433484, respectively, in the Sequence Read Archive (SRA). The reconstructed genome from ERR433484 was also deposited in the International Nucleotide Sequence Database Collaboration (INSDC) under the accession number AZEN01. Our preliminary assessment suggested that the genome AZEN01 was contaminated with *L. kefiri*. Indeed, housekeeping genes that normally exist in single copies, such as *pheS* and *rpoA*, were found in duplicate, with one matching the sequence of *L. parakefiri* deposited in the public sequence database and the other matching that of *L. kefiri*. Therefore, the only publicly available genome for the type strain of *L. parakefiri* does not serve as a reference for taxonomic studies. In this study, we obtained a type strain of *L. parakefiri*, JCM 8573^T, from the Japan Collection of Microorganisms (JCM) and reanalyzed its taxonomic status by conducting whole-genome sequencing.

Genomic DNA of JCM 8573^T was extracted from cells cultured in de Man, Rogosa, and Sharpe broth (Difco) at the mid-logarithmic phase and then purified using Qiagen Genomic-tip 500/G gravity-flow, anion-exchange tips and a Qiagen Genomic DNA Buffer Set with lysozyme (Sigma) and proteinase K (Qiagen), according to the manufacturer's instructions. Whole-genome sequencing using a 300-bp pair-end Illumina MiSeq system yielded 5,829,866 reads, which corresponds to approximately 700-fold coverage. *De novo* assembly was performed using the Platanus_B assembler (version 1.1.0) with the default settings after preprocessing the raw reads to remove low-quality bases and adapter sequences using Platanus_trim (version 1.0.7) [16]. The draft genome was annotated using the DDBJ Fast Annotation and Submission Tool (DFAST, <http://dfast.nig.ac.jp>). We obtained a draft genome that consisted of 161 contigs with an estimated genome size of 2,493,412 bp, which was comparable with those of other species in the *L. buchneri* subgroup. The genome completeness and contamination values were also calculated using CheckM (version 1.0.5) by inspecting for the presence/absence of single-copy gene markers conserved in the genus *Lactobacillus* [17]. For comparison, two data sets of raw reads, SRR1151226 and ERR433484, were downloaded from the SRA and

assembled *de novo* in the same manner. The genome statistics are summarized in Table 1. Our data showed a high completeness value and a low contamination level. Contrarily, the data from SRR1151226 and ERR433484 showed high contamination values (14.20% and 99.35%, respectively) and high ANI values (97.9% and 91.7%, respectively) against the publicly available draft genome of *L. kefiri* DSM 20587^T (accession number: AYYV01), supporting our previous findings of contamination in the sequencing data deposited in the public databases. The different genome sizes and contamination values implied different extents of contamination in the SRR1151226 and ERR433484 data. We identified one copy each of *rpoA*, *pheS*, and *recA* genes in the genome of JCM 8573^T, whose nucleotide sequences exactly matched the ones reported for *L. parakefiri* LMG 15133^T (accession numbers: AM087851, AM263510.1, and AJ621665, respectively), while showing only 92%, 84%, and 82% nucleotide identity with those of *L. kefiri* LMG 9480^T (AM087840, AM263508, and AJ621650, respectively). This strongly suggests that the strain JCM 8573^T belongs to a species distinct from *L. kefiri*. Notably, full-length 16S rRNA genes could not be identified in the draft genome. Bacterial genomes generally harbor multiple copies of rDNA regions, and such repetitive sequences make genome assembly difficult, often yielding collapsed or fragmented contigs for such regions [18]. We found that several contigs contained fragmented 16S rDNA gene sequences, which showed nearly 100% identity with the ones deposited in the public databases for *L. parakefiri*.

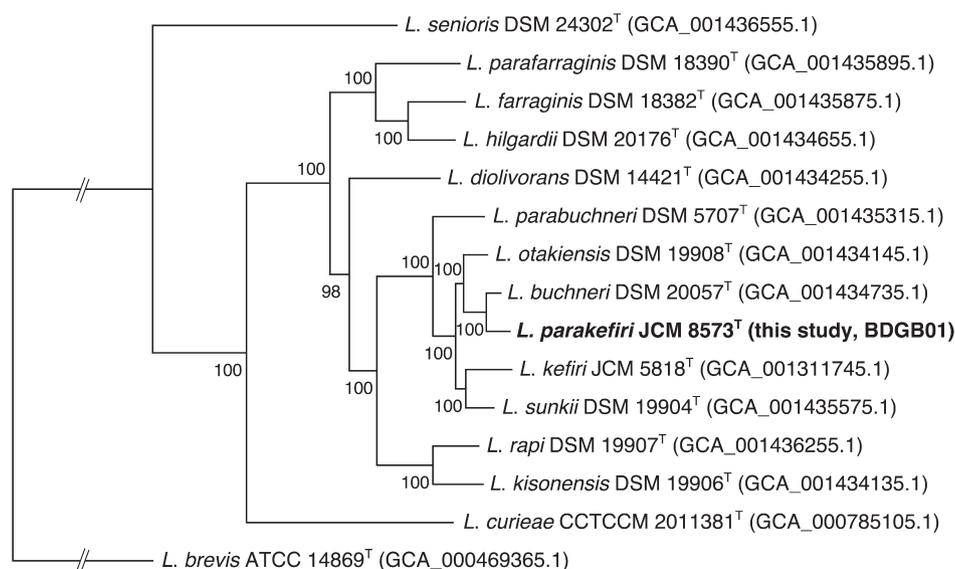
The draft genome sequence of *L. parakefiri* JCM 8573^T was deposited in the INSDC under the accession numbers BDGB01000001–BDGB01000161. The raw sequencing reads were also deposited in the SRA under the accession number DRR064132.

Next, ANI and GGD calculation were performed between *L. parakefiri* JCM 8573^T and each type strain of the 13 species in the *L. buchneri* subgroup. Genomes included in the analysis were obtained from the DFAST Archive of Genome Annotation (DAGA) [8]. ANI was calculated using the modified pyani script provided at <https://github.com/widdowquinn/pyani>, and the dDDH values were calculated using the GGDC web service (<http://ggdc.dsmz.de>). All the ANI values were less than the 95% cutoff line used to delineate two species [2], with the highest value being 84.92% against *L. buchneri* DSM 20057^T. All the dDDH values were also well below 70%. Together, these results (summarized in Table 2) support the phylogenetic position of *L. parakefiri* as a distinct species.

Figure 1 shows a phylogenetic tree constructed

Table 1. Genome statistics of *Lactobacillus parakefiri* from different data sources

Data source /Strain	Total size/ bp	Contigs	GC %	Completeness %	Contamination %	ANI against <i>L. kefiri</i> DSM 20587 ^T	
BDGB01 /JCM 8573 ^T	2,493,412	161	43.6	98.71	0.81	79.7%	Newly sequenced
SRR1151226 /DSM 10551 ^T	2,928,489	456	41.9	98.06	14.20	97.9%	Newly reconstructed
ERR433484 /DSM 10551 ^T	4,903,546	318	42.6	99.03	99.35	91.7%	Newly reconstructed
AZEN01 /DSM 10551 ^T	4,872,317	421	42.6	99.03	98.07	99.9%	INSDC data, reconstructed from ERR433484

Fig. 1. Maximum likelihood phylogenetic tree of lactobacilli in the *Lactobacillus buchneri* subgroup constructed based on multiple alignments of protein sequences of 465 single-copy genes.

L. brevis is included as the outgroup. The numbers at the internal nodes represent the bootstrapping values (1,000 replications).

Table 2. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values between *L. parakefiri* JCM 8573^T and each type strain of closely related species

Strain	Data source	ANI	dDDH
<i>L. buchneri</i> DSM 20057 ^T	GCA_001434735.1	84.92%	29.4%
<i>L. curieae</i> CCTCC M 2011381 ^T	GCA_000785105.1	72.24%	20.1%
<i>L. diolivorans</i> DSM 14421 ^T	GCA_001434255.1	74.87%	21.5%
<i>L. farraginis</i> DSM 18382 ^T	GCA_001435875.1	73.85%	20.6%
<i>L. hilgardii</i> DSM 20176 ^T	GCA_001434655.1	74.05%	20.9%
<i>L. kefiri</i> JCM 5818 ^T	GCA_001311745.1	79.71%	23.1%
<i>L. kisonensis</i> DSM 19906 ^T	GCA_001434135.1	75.16%	20.9%
<i>L. otakiensis</i> DSM 19908 ^T	GCA_001434145.1	80.62%	23.6%
<i>L. parabuchneri</i> DSM 5707 ^T	GCA_001435315.1	78.33%	21.7%
<i>L. parafarraginis</i> DSM 18390 ^T	GCA_001435895.1	74.30%	21.9%
<i>L. rapi</i> DSM 19907 ^T	GCA_001436255.1	75.12%	19.8%
<i>L. senioris</i> DSM 24302 ^T	GCA_001436555.1	71.30%	19.3%
<i>L. sunkii</i> DSM 19904 ^T	GCA_001435575.1	79.57%	22.7%

based on the conserved genes among the *L. buchneri* groups. The protein sequences of 14 type strains in the *L. buchneri* group and *L. brevis* DSM 20054^T (as an outgroup) were subjected to analysis with the GET_HOMOLOGUES software (version 1.3) [19], and 465 single-copy orthologous genes shared by all the strains were selected for phylogenetic reconstruction. The amino acid sequences within each cluster were aligned using MUSCLE (version 3.8.31) [20]. Poorly aligned or divergent regions were trimmed using Gblocks [21], and conserved regions were then concatenated using FASconCAT-G [22]. A partitioned maximum likelihood analysis was performed to construct the phylogenetic tree with RAxML (version 8.1.22) [23] using the best-fit evolutionary models predicted for each alignment by ProtTest [24]. The number of bootstrap replicates was set at 1,000. All the species were well separated with high bootstrap support values. The most closely related species of *L. parakefiri* is *L. buchneri*, which is in agreement with the ANI and dDDH results as well as the phylogenetic tree constructed based on the 16S rRNA gene sequences [25], although the tree topology is slightly different.

All these results confirmed the taxonomic position of *L. parakefiri* and indicated no contamination of *L. kefir* in the type strain deposited in JCM. According to the cell history of JCM 8573^T (<http://www.straininfo.net/strains/54579>) [9], it was directly deposited in the JCM from a subculture of the original isolate, GCL 1731^T, and then transferred to other culture collections, including the German Collection of Microorganisms and Cell Cultures (DSMZ). Hence, the JCM strain is most unlikely to contain contamination. To examine whether the DSMZ strain contains contamination, we also obtained the cell line of *L. parakefiri* DSM 10551^T. We randomly picked 16 colonies from cells cultured on de Man, Rogosa, and Sharpe broth and sequenced their 16S rRNA genes using the primers described previously [26]. All sequences were identical to that of *L. parakefiri* JCM 8573^T (accession number: LC096211). In addition, PCR amplification of partial *pheS* genes was conducted by using two sets of primers, each of which were specific for *L. parakefiri* and *L. kefir*. Briefly, genomic DNAs of all tested strains were prepared as described above. Primers for specific amplification of partial *pheS* genes of *L. parakefiri* or *L. kefir* were designed: for *L. parakefiri*, LPKF, AGACCAGCGACTTAAAGGCA, and LPKR, CCAACGTTTCTCGATGATTC, which amplified a 186-bp fragment, and for *L. kefir*, LKF, CAAACCAACGACTTGAAGAAG, and LKR, CAAGCTTTTGGCGTTGATCG, which amplified a 187-bp fragment. The reactions were performed with a

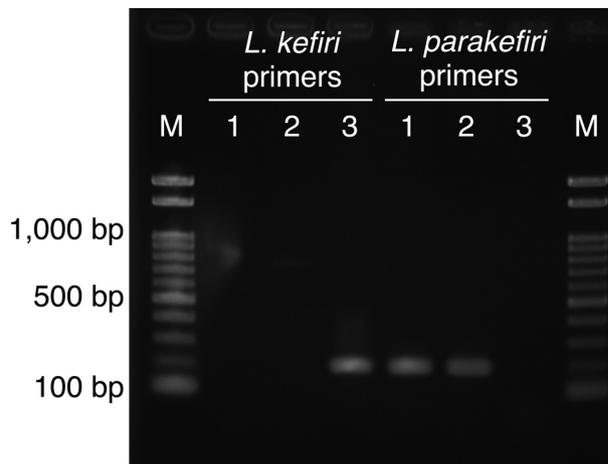


Fig. 2. Amplification products obtained from a *pheS* PCR assay using the *L. kefir*- and *L. parakefiri*-specific primers.

Lanes: M, Gene Ladder 100 (Wako Pure Chemical Industries, Ltd.); 1, *L. parakefiri* DSM 10551^T; 2, *L. parakefiri* JCM 8573^T; 3, *L. kefir* JCM 5818^T.

GeneAmp PCR System 9700 (Thermo Fisher Scientific) using EmeraldAmp PCR Master Mix (TaKaRa) according to the manufacturer's instructions. The reaction was initiated by denaturation for 30 sec at 98°C, followed by 40 cycles of 10 sec at 98°C, 30 sec at 60°C, and 30 sec at 72°C. As shown in Fig. 2, an amplified product was obtained from the DSMZ sample only when the *L. parakefiri*-specific primers were used, indicating no evidence of contamination with *L. kefir*. Therefore, the reason for contamination in both the SRR1151226 and ERR433484 data, for which DSM 10551^T was used in genome sequencing, remains unclear.

In this study, we report the first reliable genome for *L. parakefiri*. The genome contains 2,444 protein-coding sequences (CDSs). It has been reported that *L. parakefiri* produces gas from glucose but not from gluconate [9]. This unusual trait is likely due to the frameshift mutation that resulted in a premature stop codon in the coding region of gluconate permease, which is located adjacent to the gluconate kinase gene. In other heterofermentative species, including *L. buchneri* and *L. kefir*, the two genes are thought to take roles in uptake and phosphorylation of gluconate, constituting an initial stage of its metabolic pathway. Other metabolic gene contents of *L. parakefiri* are similar to those of its most closest relative, *L. buchneri*. They share homologues of both lactaldehyde dehydrogenase and lactaldehyde reductase involved in the anaerobic lactate degradation to acetate and 1,2-propanediol. This characteristic property of *L. buchneri* is important for the dairy industry, because

acetate enhances the aerobic stability of silage after exposure to air by preventing the growth of molds and yeasts [27, 28]. JCM 8573^T also possesses two genes responsible for the conversion of serine to cysteine (serine O-acetyltransferase and cysteine synthase A), which differentiates it from *L. buchneri* and *L. kefir* that do not possess these genes. Interestingly, as many as 10 of the predicted CDSs were shared by *L. kefiranofaciens* subsp. *kefirgranum*, a rather distantly related homofermentative lactobacillus, with a nucleotide identity of more than 99%. This number exceeds that for the more closely related *L. kefir* (6 CDSs) detected under the same condition. *L. kefiranofaciens* subsp. *kefirgranum* was first isolated from kefir grains together with *L. parakefiri* by the same authors [9]. The high nucleotide identity may indicate the recent acquisition of these genes in similar biological environments. These include genes annotated as major facilitator superfamily transporter, nicotinate-nucleotide pyrophosphorylase, or hypothetical protein. It will be interesting to speculate how these genes have affected the coevolution of the two species and how they function in the microbiome of the kefir grain. The genome information we provide here may be used as a reference, especially for taxonomic studies, as well as for biotechnological applications of kefir-grain microbial flora.

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