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Detection and Genomic Characterization of Motility in *Lactobacillus curvatus*: Confirmation of Motility in a Species outside the *Lactobacillus salivarius* Clade

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Lactobacillus **is the largest genus within the lactic acid bacteria (LAB), with almost 180 species currently identified. Motility has been reported for at least 13** ***Lactobacillus*** **species, all belonging to the** ***Lactobacillus salivarius*** **clade. Motility in lactobacilli is poorly characterized. It probably confers competitive advantages, such as superior nutrient acquisition and niche colonization, but it could also play an important role in innate immune system activation through flagellin–Toll-like receptor 5 (TLR5) interaction. We now report strong evidence of motility in a species outside the** ***L. salivarius*** **clade,** ***Lactobacillus curvatus*** **(strain NRIC 0822). The motility of** ***L. curvatus*** **NRIC 0822 was revealed by phase-contrast microscopy and soft-agar motility assays. Strain NRIC 0822 was motile at temperatures between 15°C and 37°C, with a range of different carbohydrates, and under varying atmospheric conditions. We sequenced the** ***L. curvatus*** **NRIC 0822 genome, which revealed that the motility genes are organized in a single operon and that the products are very similar (>98.5% amino acid similarity over >11,000 amino acids) to those encoded by the motility operon of** ***Lactobacillus acidipiscis*** **KCTC 13900 (shown for the first time to be motile also). Moreover, the presence of a large number of mobile genetic elements within and flanking the motility operon of** ***L. curvatus*** **suggests recent horizontal transfer between members of two distinct** ***Lactobacillus*** **clades:** ***L. acidipiscis*** **in the** ***L. salivarius*** **clade and** ***L. curvatus*** **in the** ***L. sakei*** **clade. This study provides novel phenotypic, genetic, and phylogenetic insights into flagellum-mediated motility in lactobacilli.**

Motility in bacterial species is often mediated by a sophisticated molecular structure called the flagellum. This chief organelle of bacterial motility is self-assembled using dozens of different proteins and rotates to propel the cell forward (1). The filament of the bacterial flagellum is composed of one or more flagellin proteins, a microbe-associated molecular pattern (MAMP) which is recognized by the host Toll-like receptor 5 (TLR5) (2) and which, via activation of the nuclear factor kappa B (NF-κB) signaling pathway, engages defense responses both systemically and at epithelial surfaces (3). Several flagellate bacterial pathogens (alphaproteobacteria and epsilonproteobacteria) have evolved flagellin proteins with sequence changes that avoid TLR5 recognition while maintaining motility (4). In an ecosystem, flagellum-mediated motility may confer a competitive advantage on motile species over nonmotile species with respect to niche colonization, biofilm formation, and the secretion of virulence proteins by pathogenic bacteria (5).

Lactobacillus spp. constitute a very diverse group and the largest genus within the lactic acid bacteria (LAB). Lactobacilli are associated mainly with food production and probiotics (6). Some *Lactobacillus* species colonize the gastrointestinal, oral, and genital tracts of humans, making them important members of the human microbiota (7). The genus *Lactobacillus* has been widely researched because of its importance for health and food applications. However, to date, motility in lactobacilli is poorly characterized. The motility of flagellate species has not attracted much scientific consideration, leading to the continued perception that the *Lactobacillus* genus is nonmotile (8–10). To date, at least 13 motile species have been officially recognized in the genus *Lactobacillus* (5, 11), and all of them belong to the *Lactobacillus salivarius* clade (Table 1) (12). The motility of *L. ruminis* has been particularly well studied previously in strain ATCC 27782 at both the phenotypic and genomic levels (5). In this strain, all the genes required to produce a fully functional flagellar apparatus have been identified and have been shown to be organized in a single operon (5, 13).

L. curvatus is one of the LAB most commonly associated with fermented meat goods, vacuum-packaged refrigerated meat, and, to a lesser extent, ready-to-eat meat, fish, and poultry products (14). *L. curvatus* is a member of the *L. sakei* clade (based on 16S rRNA gene phylogeny) and is phylogenetically related to the species *L. sakei*, *L. fuchuensis*, and *L. graminis* (12), all of which are associated with meat environments. To date, the only genome sequence available for *L. curvatus* is that of strain CRL705 (14),

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TABLE 1 Origin and phylogeny of motile *Lactobacillus* species described to date

Species	Strain	Source	Type strain	Clade[a]	Reference(s) for motility
L. acidipiscis	KCTC 13900	Halloumi cheese made from ovine milk	Yes[b]	*L. salivarius*	This study
L. agilis	DSM 20509^T	Municipal sewage	Yes	*L. salivarius*	5, 48
L. aquaticus	DSM 21051^T	Korean freshwater pond	Yes	*L. salivarius*	5, 49
L. cacaonum	DSM 21116^T	Cocoa bean heap fermentation	Yes	*L. salivarius*	This study
L. capillatus	DSM 19910^T	Fermented stinky tofu brine	Yes	*L. salivarius*	5, 50
L. curvatus	NRIC 0822	Kabura-zushi	No	*L. sakei*	This study
L. ghanensis	DSM 18630^T	Cocoa fermentation	Yes	*L. salivarius*	5, 51
L. hordei	DSM 19519^T	Malted barley	Yes	*L. salivarius*	This study
L. mali	DSM 20444^T	Apple juice from cider press	Yes	*L. salivarius*	5, 52
L. nagelii	DSM 13675^T	Commercial red wine	Yes	*L. salivarius*	5, 53
L. oeni	DSM 19972^T	Bobal grape wine	Yes	*L. salivarius*	5, 54
L. ruminis	ATCC 27782	Bovine rumen	No	*L. salivarius*	5, 55
L. satsumensis	DSM 16230^T	Mashes of shochu	Yes	*L. salivarius*	5, 56
L. sicerae	CECT 8227^T	Ropy natural cider	Yes	*L. salivarius*	11
L. sucicola	DSM 21376^T	Sap of oak tree	Yes	*L. salivarius*	5, 57
L. uvarum	DSM 19971^T	Bobal grape musts	Yes	*L. salivarius*	5, 58
L. vini	DSM 20605^T	Grape must, fermenting at high temp	Yes	*L. salivarius*	5, 59

[a] According to the phylogeny of Salvetti et al., 2012 (12).
[b] Type strain of *Lactobacillus cypricasei* (36).

isolated from an Argentinean artisanal fermented sausage and well known for bacteriocin production (15).

In this study, we provide strong evidence of motility in a *Lactobacillus* species outside the *L. salivarius* clade, *L. curvatus* (strain NRIC 0822). We assessed this motility behavior under different environmental conditions and confirmed the presence of motility genes organized in a single operon. We also identified *L. acidipiscis* KCTC 13900 as a motile strain, which had not been shown before, and we identified two other *Lactobacillus* species (*L. cacaonum* and *L. hordei*), also belonging to the *L. salivarius* clade, as potentially motile. The gaps in 16 motility loci that were present on different contigs in the draft genome assemblies of *Lactobacillus* spp. were closed by PCR and sequencing to allow a global comparison of the motility loci of this genus. We also analyzed the phylogeny of *Lactobacillus* motility proteins and found it to be congruent with the 16S rRNA gene-based phylogeny of the species in the *L. salivarius* clade.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and motility evaluation. *Lactobacillus* strains (Table 1) were routinely cultured in MRS medium at 37°C or 30°C either anaerobically, aerobically, or under 5% CO_2, depending on the strain.

Strain NRIC 0822, isolated in Japan from kabura-zushi, a fermented archetype of sushi (16), was identified as *L. curvatus* by Gram staining, catalase testing, CO_2 production from glucose, lactic acid isomer production, and carbohydrate fermentation, as well as by sequencing of the 16S rRNA gene. This identification was confirmed again after genome sequencing, (i) by BLAST analysis of the nucleotide sequences of the *pheS*, *rpoA*, *tuf*, *atpA*, and *hsp60* genes against the NCBI nonredundant (nr) database and (ii) by whole draft genome comparisons using two DNA-DNA hybridization (DDH) prediction models: average nucleotide identity (ANI [17]) and genome-to-genome distance calculator (GGDC [18]) values.

Standard motility agar assays were used to evaluate the influence of atmospheric conditions (aerobic, anaerobic, 5% CO_2), temperature (10°C, 15°C, 20°C, 25°C, 30°C, 37°C, and 42°C), and carbohydrates (glucose, galactose, fructose, mannose, and maltose, which had been identified as the carbohydrates fermented by strain NRIC 0822 during its identification) on the motility of *L. curvatus* NRIC 0822. A carbohydrate-free MRS medium (cfMRS), in which the carbohydrate source (glucose) and meat extract were omitted as described previously (19), was used as a basal growth medium. Motility under the different atmospheric conditions was assessed in cfMRS broth supplemented with 0.5% glucose and 0.3% to 0.5% (wt/vol) agar (soft agar) at 30°C. Six-well plates (Corning Incorporated) containing 8 ml of soft agar were inoculated (by stabbing) with 5 μl of an overnight culture of the strain to be tested. The plates were allowed to dry for 3 min and were incubated for a maximum of 48 h. Test tubes containing 17 ml of soft agar were inoculated with a needle previously plunged into an overnight culture. The tubes were allowed to dry for 3 min and were incubated as described above. The same procedure was used to test the motility of *L. curvatus* NRIC 0822 (i) at different temperatures in cfMRS containing 0.5% glucose under anaerobic conditions and (ii) in the presence of different carbohydrates in cfMRS containing 0.5% of the test carbohydrate at 30°C under 5% CO_2.

For testing the motility of *L. curvatus* NRIC 0822, three control strains were always included. *L. ruminis* ATCC 27782 was used as a positive control, since its motility and genome sequence have been described well previously (5, 13). The previously sequenced *L. acidipiscis* strain KCTC 13900 (20) was used because its genome harbors the top BLAST hit for all motility genes in *L. curvatus* NRIC 0822. *L. curvatus* DSM 20019^T was used as a negative control for motility, because its genome does not contain motility genes.

All the experiments were carried out in biological triplicate. Images of each of the 6-well plates and test tubes were taken using the GeneGenius bioimaging system (Syngene) and an Xperia Z camera (Sony), respectively.

Culture motility was also evaluated by phase-contrast microscopy as described previously (5). Briefly, glass capillary tubes were first filled with an aliquot of the bacterial culture and then placed on a heated microscope stage that was maintained at 30 to 37°C (according to the strain growth conditions) for the evaluation of culture motility. When every bacterium in a field of view of the microscope was either running or tumbling and moving quickly, the culture was considered "motile." If no motile bacteria were observed in the fields of view examined, the culture was considered "nonmotile."

Chemotaxis was also assessed by a chemical-in-plug assay. In this assay, a solid (1.5%) agar plug (diameter, about 7 mm) containing the chemical to be tested was placed in the center of a petri dish. A soft (0.2%)

MRS agar medium without yeast extract (prewarmed at 48°C) was inoculated (0.05%) with an overnight culture, and 20 ml was poured around the agar plug. The plates were allowed to dry for 10 min and were incubated for a maximum of 48 h at 30°C under 5% CO_2. The presence of an outer chemotactic ring indicated positive results for chemotaxis.

Microscopy. For transmission electron microscopy (TEM), *L. curvatus* NRIC 0822 cells were added to an equal volume of 5% glutaraldehyde, and the container was gently inverted to mix the contents. Cells were allowed to sediment overnight at 4°C. The supernatant was removed, and each cell pellet was resuspended in 2.5% glutaraldehyde and was flicked briefly to mix. Five microliters of the cell suspension was added to Formvar carbon-coated 200-mesh copper grids and was incubated at room temperature for 5 min. Liquid was wicked away by touching the side of the grid with a sheet of clean filter paper. Grids were washed by quick dipping in filtered sterile water and were dried by wicking. Five microliters of uranyl acetate (0.5%) was added to the grids for 5 min. Liquid was wicked away, and the grids were allowed to dry for 5 min. Grids were imaged by transmission electron microscopy under a vacuum.

For light microscopy, *L. curvatus* NRIC 0822 cells were prestained with a Flagella Reagent Dropper (Becton Dickinson Microbiology Systems) according to the manufacturer's instructions.

Genome sequencing and annotation of *L. curvatus* NRIC 0822. Paired-end reads were obtained using the Illumina HiSeq 2000 reversible dye terminator system (Macrogen, Seoul, South Korea), with read lengths of 101 bp. Sequencing generated 29,761,714 reads (3,005,933,114 bp). *De novo* genome assembly of the Illumina sequences was performed using Velvet (version 1.2.07 [21]), producing an assembly of 144 contigs These 144 assembled contigs represent 1,417-fold genome coverage based on an estimated genome size of 1.94 Mb. The N_{50} score for the assembly was 25,925 bp. Automated gene calling was performed using Glimmer, version 3 (22). tRNA genes were identified using tRNAscan (version 1.23 [23]). All predicted proteins were searched (BLASTP) against the NCBI nonredundant (nr) protein database.

DNA extraction and gap closure. After 24 to 48 h of culture, the genomic DNA of *Lactobacillus* species positive for motility genes was isolated by using the Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions for Gram-positive bacteria. The genomic DNA was quantified using a spectrophotometer (NanoDrop 2000; Thermo Scientific) and was checked for integrity on a 0.8% agarose gel.

A PCR-based strategy was adopted for gap closure in the motility operons. Contig-contig gaps were closed using primers designed at the beginnings (reverse strand) and ends (forward strand) of contigs, and these regions were amplified using Phusion Hot Start II polymerase (Thermo Scientific). Contigs were ordered and oriented by PCR. A two-step walking PCR method (24) was also used to amplify the upstream or downstream contig regions when they were unknown, in order to check for the presence or absence of mobile genetic elements flanking the motility operon. Primers were designed using the Primer3Plus Web tool. Purified PCR products for both closing gaps and walking PCRs were sequenced by GATC Biotech (Cologne, Germany). Once the gaps were closed, genes in the motility operons were predicted as described above.

***In silico* confirmation and identification of motile *Lactobacillus* species.** Protein sequence motifs for each of five motility components were identified using the available draft genomes of *L. curvatus* NRIC 0822, *L. ruminis* ATCC 27782, *L. acidipiscis* KCTC 13900, *L. mali* DSM 20444^T, and *L. vini* DSM 20605^T. The motifs identified corresponded to different regions of the motility operons and encompassed the flagellin FliC, the flagellar hook-basal body complex protein FliE, the flagellar motor switch protein FliG, the flagellar biosynthesis protein FlhA, and the chemotaxis protein MotA (see Table S1 in the supplemental material). A TBLASTN search for these 5 motifs was performed against all publicly available *Lactobacillus* sp. complete and draft genomes, as well as against the *Lactobacillus* sp. draft genomes that were recently sequenced as part of the *Lactobacillus* genome-sequencing initiative (Z. Sun, H. M. B. Harris, A. McCann, X. Yang, S. Argimon, W. Zhang, C. Guo, I. B. Jeffery, J. C. Cooney, T. F. Kagawa, W. Liu, Y. Song, E. Salvetti, A. Wrobel, P. Rasinkangas, J. Parkhill, M. C. Rea, O. O'Sullivan, J. Ritari, F. P. Douillard, R. P. Ross, R. Yang, A. Briner, G. Felis, W. M. de Vos, R. Barrangou, T. R. Klaenhammer, P. W. Caufield, Y. Cui, H. Zhang, and P. W. O'Toole, submitted for publication), for a total of 349 genomes. The *L. sicerae* species, described as motile (11), was not included in this study, because this new species was described too recently, and the partial draft genome did not allow us to identify all the motility genes.

Alignments and phylogenetic analyses. The sequence of the 16S rRNA gene of the type strain of each *Lactobacillus* species was downloaded from the NCBI GenBank database. A maximum likelihood (ML) phylogeny was constructed in MEGA6 (25) from a MUSCLE alignment with the appropriate substitution model for the ML option selected (26) and 1,000 bootstrap replications. The phylogenetic tree shown in Fig. 5 was made online using the Interactive Tree Of Life (27).

Start codons were manually corrected by using the average length of the sequences for each protein to indicate the most likely start site and by looking for the presence of a ribosome binding site (RBS) motif upstream. This was a necessary step, because gene prediction software is often inaccurate at predicting start sites for genes. Motility protein sequences were then concatenated. Alignment and phylogenetic tree construction were carried out as described above.

Protein identity and similarity scores were obtained from the LALIGN Web tool (28), using alignment default values.

Nucleotide sequence accession numbers. The BioProject identification code (ID) for this study is PRJNA265031. This whole-genome shotgun project for *L. curvatus* NRIC 0822 has been deposited at DDBJ/EMBL/GenBank under accession number JTJV00000000. The version described in this paper is version JTJV01000000. The GenBank accession number for the full-length 16S rRNA gene of *L. curvatus* NRIC 0822 is KM676454. The GenBank accession numbers for the 16 individual motility operons are KM886858 to KM886873 (KM886858 for *L. acidipiscis* KCTC 13900, KM886859 for *L. agilis* DSM 20509^T, KM886860 for *L. aquaticus* DSM 21051^T, KM886861 for *L. cacaonum* DSM 21116^T, KM886862 for *L. capillatus* DSM 19910^T, KM886863 for *L. curvatus* NRIC 0822, KM886864 for *L. ghanensis* DSM 18630^T, KM886865 for L. *hordei* DSM 19519^T, KM886866 for *L. mali* DSM 20444^T, KM886867 for *L. nagelii* ATCC 700692^T, KM886868 for *L. oeni* DSM 19972^T, KM886869 for *L. ruminis* ATCC 27782, KM886870 for *L. satsumensis* DSM 16230^T, KM886871 for *L. sucicola* DSM 21376^T, KM886872 for *L. uvarum* DSM 19971^T, and KM886873 for *L. vini* DSM 20605^T).

RESULTS

***L. curvatus* NRIC 0822 employs flagellum-mediated motility.** Strain NRIC 0822 was isolated from kabura-zushi, a Japanese fermented food made from rice, turnip, and fish. This strain was observed to be motile in our routine lab tests and was identified as *L. curvatus* by 16S rRNA gene sequencing. To investigate the motility of *L. curvatus* NRIC 0822, phase-contrast microscopy and standard motility agar assays were conducted. Under microscopy, this strain displayed moderate motility in the exponential phase in MRS broth, demonstrating swimming and tumbling motilities. *L. curvatus* NRIC 0822 was also observed to be motile in both soft agar assays used (test tubes and 6-well plates) (Fig. 1A). The cultures of *L. curvatus* NRIC 0822 were nonmotile in the stationary phase. The two positive controls, *L. ruminis* ATCC 27782 and *L. acidipiscis* KCTC 13900, were also motile, whereas *L. curvatus* DSM 20019^T, the type strain of the species, was nonmotile. Preliminary investigation demonstrated positive chemotaxis for *L. curvatus* NRIC 0822 but not for the nonmotile *L. curvatus* strain DSM 20019^T (Fig. 1B). As expected, enhanced growth was observed for both motile and nonmotile *L. curvatus* strains around the plug containing yeast extract. However, a sharp ring of concentrated cells (a typical chemotactic ring) specific for the me-

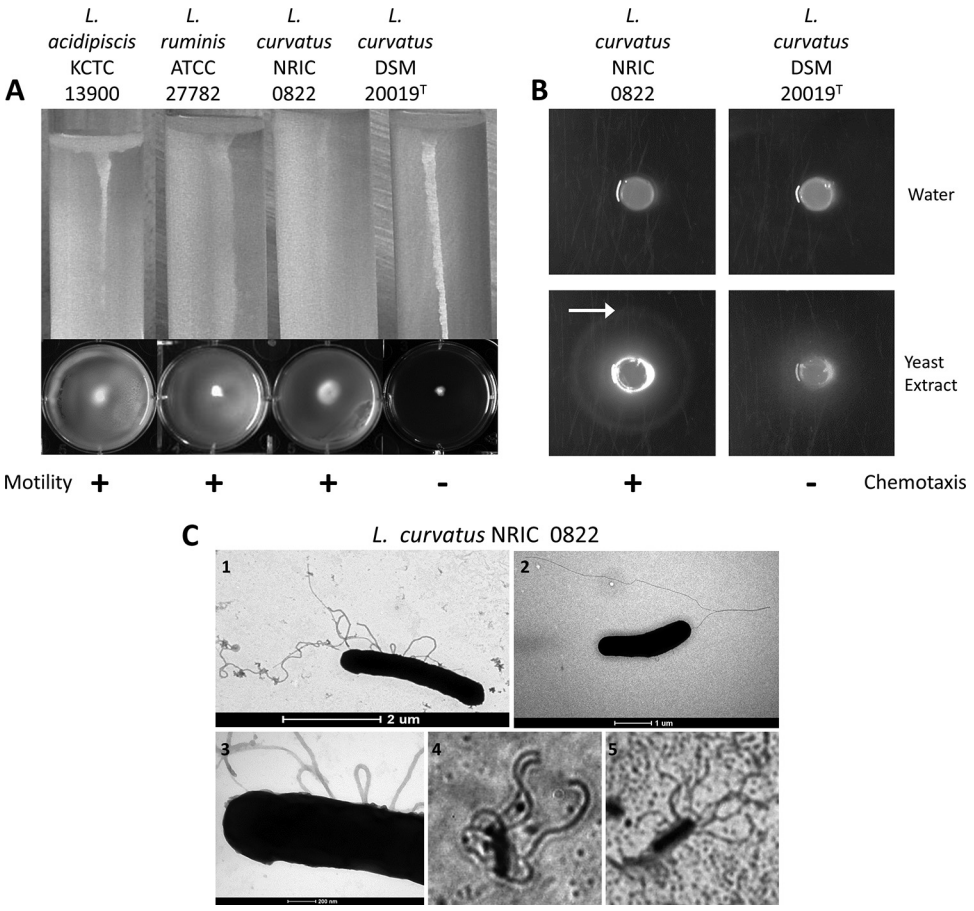

FIG 1 Motility and flagella of *L. curvatus* NRIC 0822. (A) Soft-agar assays in tubes (top) and 6-well plates (bottom) showing the motility of *L. curvatus* NRIC 0822. Three controls were included: *L. ruminis* ATCC 27782 (positive control for motility), *L. acidipiscis* KCTC 13900 (top BLAST hit for all the motility genes in *L. curvatus* NRIC 0822), and *L. curvatus* DSM 20019^T (negative control for motility). (B) Chemotaxis phenotype of *L. curvatus* NRIC 0822 for yeast extract. Chemotaxis was assessed by a chemical-in-plug assay, with sterile water or yeast extract (2.5%) as the test compound. The arrow indicates the location of the outer chemotactic ring, indicating a positive result for chemotaxis. (C) Images of an *L. curvatus* NRIC 0822 whole cell and flagella. (Panels 1 to 3) Transmission electron micrographs (magnifications, ×8,200, ×11,500, and ×60,000, respectively); (panels 4 and 5) light microscopy pictures after flagellum staining (magnification, ×1,000).

dium components in the plug and plate was present only with *L. curvatus* NRIC 0822, and not with water. Thus, this strain sensed and responded positively chemotactically to the chemical stimulus. To further investigate the flagellate phenotype of *L. curvatus* NRIC 0822, TEM and flagellar staining were performed, revealing the presence of flagella with a peritrichous organization (Fig. 1C). The peritrichous flagella are more visible with the light microscope after flagellar staining than on the TEM images, where the flagella look sparse. This is probably due to fixation during the preparation of cells for TEM, which altered the fragile flagellar filaments. In addition, the effects of different environmental conditions (atmospheric conditions, temperature, and carbohydrate sources) on the motility of *L. curvatus* NRIC 0822 were investigated, and the results were consistent throughout the triplicate experiments. *L. curvatus* NRIC 0822 was motile under 5% CO_2, as well as under anaerobic and aerobic conditions, in both the 6-well plates and the test tubes (Table 2). Motility was also observed at temperatures ranging from 15 to 37°C (Table 2). *L. curvatus* NRIC 0822 grew at 10°C and 42°C, but no motility was observed. *L. curvatus* NRIC 0822 was also motile when grown with all five carbohydrates tested in both test tubes and 6-well plates (Table 2).

Genome sequencing of *L. curvatus* NRIC 0822 confirms the presence of motility genes organized in a single operon. The draft genome of *L. curvatus* NRIC 0822 comprised 144 contigs, for a total assembly size of 1.94 Mbp, an N_{50} value of 25,925 bp, and 1,417-fold coverage (see Table S2 in the supplemental material). The draft genome of *L. curvatus* NRIC 0822 includes 1,944,912 bases (GC content, 41.70%). It comprises 2,060 predicted genes or coding DNA sequences (CDS), and 56 predicted tRNAs, representing all 20 amino acids, were identified in the genome. The genome of *L. ruminis* ATCC 27782 had been sequenced in our laboratory previously (13) and was used to direct our study of *L. curvatus* NRIC 0822 and the organization of its motility genes. Annotation of the *L. curvatus* NRIC 0822 draft genome identified motility genes spread over 8 contigs in the draft assembly. The sequences of these contigs were joined by PCR and sequencing. Walking PCR was also used on the flanking regions in order to confirm the limit of motility-related (or mobile genetic element-related) sequences. Among the genome sequences of *L. curvatus* available so far, the presence of motility genes is a unique trait of strain NRIC 0822 (Fig. 2). The genome of NRIC 0822 is otherwise nearly identical to the two other *L. curvatus* draft genomes (Fig. 2;

TABLE 2 Effects of growth conditions on the motility of *Lactobacillus curvatus* NRIC 0822

Growth conditions	Motility[a]
Atmospheric conditions[b]	
Aerobic	+
5% CO_2	+
Anaerobic	+
Temp (°C)[c]	
10	−
15	+
20	+
25	+
30	+
37	+
42	−
Carbohydrate[d]	
Glu	+
Gal	+
Fru	+
Man	+
Mal	+

[a] +, motile; −, nonmotile.
[b] With glucose at 30°C.
[c] With glucose under anaerobic conditions.
[d] At 30°C under 5% CO_2. Glu, glucose; Gal, galactose; Fru, fructose; Man, mannose; Mal, maltose.

see also Tables S2 and S3 in the supplemental material). The motility genes in *L. curvatus* NRIC 0822 are organized in a single operon of 49.3 kb, as in *L. ruminis* ATCC 27782 (Fig. 3). This motility operon is flanked by transposases, which bring the motility locus to 56 kb when included. Among the 46 motility proteins (involved in flagellum assembly, export, and chemotaxis) of *L. curvatus* NRIC 0822, 45 have a top BLASTP hit in the predicted proteins of *L. acidipiscis* KCTC 13900, and only the MCP3 protein of *L. curvatus* NRIC 0822 has no homolog (but this predicted gene seems to have been disrupted by an integrase [Fig. 3]). The sequence of the motility operon of *L. acidipiscis* KCTC 13900 (4 contigs in the publicly available draft genome in the NCBI database [GenBank Assembly ID GCA_000260635.1]) was also closed by PCR in order to allow the overall comparison of motility operons between strains. The organization of the *L. curvatus* NRIC 0822 motility operon was compared to that of the well-studied *L. ruminis* ATCC 27782 motility operon and its closest relative based on BLAST hits, *L. acidipiscis* KCTC 13900 (Fig. 3; see also Table S4 in the supplemental material). The organization of the *L. curvatus* NRIC 0822 motility operon is quite similar to that of *L. ruminis* ATCC 27782, with a major central block showing the same gene positions, whereas the beginning and end of the operon are inverted between the two strains (Fig. 3). The organization and gene content of the *L. curvatus* NRIC 0822 and *L. acidipiscis* KCTC 13900 motility operons are very similar (98.8% identity between the concatenated proteins). Over the individual proteins, similarity ranges from 96% to 100% and identity from 93% to 100% (see Table S4). The GC content was also more similar between the motility operons of these two strains (0.10% difference) than over their whole genomes (>2% difference), supporting the idea of a recent horizontal gene transfer. The *L. ruminis* ATCC 27782 and *L. curvatus* NRIC 0822 motility proteins have an average similarity of 83% (minimum, 59%; maximum, 98%) and an average identity of 54% (minimum, 23%; maximum, 87%). With regard to the gene composition of the motility operons, the LRC_16020 gene, encoding a flagellar operon protein (FOP), is present only in *L. ruminis* ATCC 27782. The *L. curvatus* NRIC 0822 genome harbors a gene encoding an additional methyl-accepting chemotaxis protein (MCP), MCP3, at the end of its motility operon (Fig. 3).

Identification of motility genes in *L. salivarius* clade genomes. Motility is described solely for members of the *L. salivarius* clade of lactobacilli. We used 5 motility protein sequence motifs (ranging from 63 to 325 amino acids) to search for homologs in the draft genomes of additional species in this clade whose genomes were recently sequenced as part of the *Lactobacillus* genome-sequencing initiative (Sun et al., submitted). All 12 species previously described as motile in the *L. salivarius* clade (Table 1) returned significant hits, based on TBLASTN searches, with all 5 motifs. In addition to the genomes of the 4 strains belonging to the *L. salivarius* clade used to design the motifs (i.e., *L. ruminis*, *L. mali*, *L. vini*, and *L. acidipiscis* strains; the latter is newly described as motile in this study), the motility motif search confirmed the presence of genes involved in motility in *L. agilis* DSM 20509^T, *L. aquaticus* DSM 21051^T, *L. capillatus* DSM 19910^T, *L. ghanensis* DSM 18630^T, *L. nagelii* DSM 13675^T, *L. oeni* DSM 19972^T, *L. satsumensis* DSM 16230^T, *L. sucicola* DSM 21376^T, and *L. uvarum* DSM 19971^T. Two additional strains, *L. cacaonum* DSM 21116^T and *L. hordei* DSM 19519^T, belonging to species that are also part of the *L. salivarius* clade, were identified as likely to be motile, because BLAST analysis using motility protein search motifs returned significant hits. The sequences of motility contigs in all of the draft genomes were joined by PCR and sequencing to allow a global comparison of the 16 motility operons.

Concordant phylogeny of motility proteins in the *L. salivarius* clade. Phylogenetic analysis was performed on the motility operons of the 16 strains listed in Table 1 (excluding *L. sicerae*), which included a total of 749 predicted genes. Three genes presented a frameshift due to a stop codon, and the corresponding proteins were corrected manually before running the alignment in order to avoid the bias that truncated proteins would have introduced into the phylogenetic analyses. These three genes were *fliI* in *L. vini* DSM 20605^T, *fliM* in *L. hordei* DSM 19519^T, and *fliP* in *L. agilis* DSM 20509^T. This phylogenetic analysis of the 16 motility operons demonstrated strong concordance with the 16S rRNA gene-based phylogenetic tree (Fig. 4). The *L. salivarius* clade can be divided into two subclades, represented by *L. mali* and *L. ruminis* (the latter subclade includes *L. salivarius*). All 13 species in the *L. mali* subclade are motile, whereas only 3 of the 15 species in the *L. ruminis* subclade are motile (Fig. 4A). The ML tree based on the concatenated motility proteins is in accordance with this division into subclades and also confirmed the strong relationship between the *L. curvatus* NRIC 0822 and *L. acidipiscis* KCTC 13900 motility operons (Fig. 4B). Moreover, the clustering of the motile *Lactobacillus* species based on the concatenated motility proteins is concordant with the 16S rRNA phylogeny (Fig. 4). This concordance between the 16S rRNA gene and concatenated motility proteins was also observed in most of the individual motility protein trees (see Fig. S1 in the supplemental material). The global identities (see Table S5 in the supplemental material) and similarities (see Table S6 in the supplemental material) between the motility operons also demonstrated the same close relationships within the *L. salivarius* clade that were shown by the 16S rRNA phylogenetic

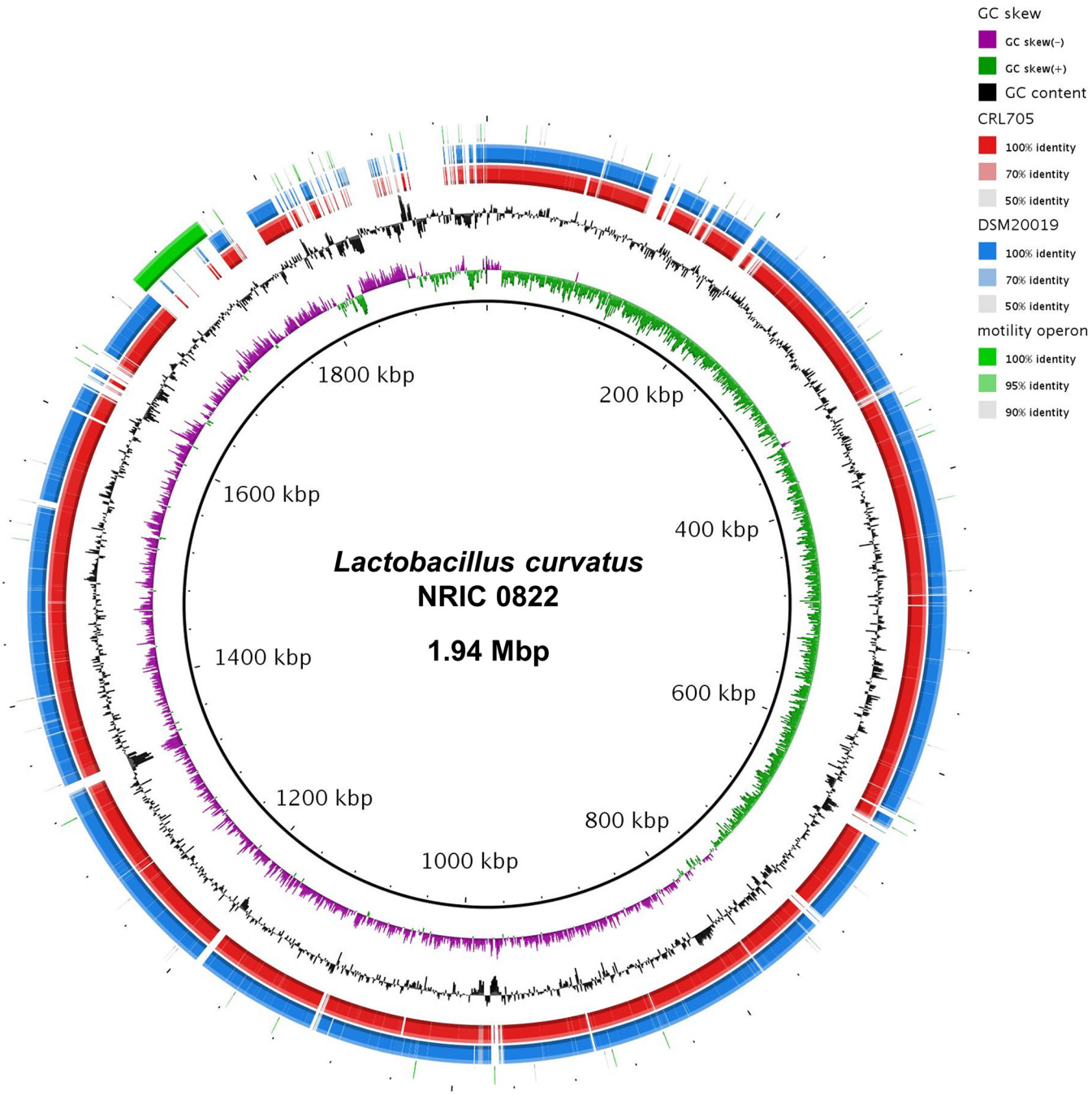

FIG 2 BLAST Ring Image Generator representation of three *L. curvatus* draft genomes. The draft reference genome of *L. curvatus* NRIC 0822 is compared to the draft genomes of the nonmotile strains *L. curvatus* DSM 20019^T (blue) and CRL 705 (red) by using the BLAST Ring Image Generator (46). The reference genome is an ordered set of contigs, based on an alignment with the closest complete genome, *L. sakei* 23K (BioProject accession no. PRJNA13435), by use of Mauve (47). The innermost rings show the GC skew (purple/green) and GC content (black). The red and blue rings show BLAST comparisons of the other two *L. curvatus* draft genomes against the *L. curvatus* NRIC 0822 draft genome assembly. The outermost arc, shown in green, highlights the motility operon of *L. curvatus* NRIC 0822.

analysis. All these data (see Fig. S1 and Tables S4, S5, and S6 in the supplemental material) confirmed again the close phylogenetic relationship between the motility operons of *L. curvatus* NRIC 0822 and *L. acidipiscis* KCTC 13900.

Motility in *Lactobacillus* spp. We also used the 5 motility protein sequence motifs to search against the draft genomes of all the *Lactobacillus* species that were recently sequenced as part of the *Lactobacillus* genome-sequencing initiative (Sun et al., submitted). No new species returned significant hits. Thus, apart from *L. curvatus* NRIC 0822, motility in lactobacilli is confined to the *L. salivarius* clade (Fig. 5). Our study resulted in a new total of 17 motile species (15 species with a motile phenotype and motility

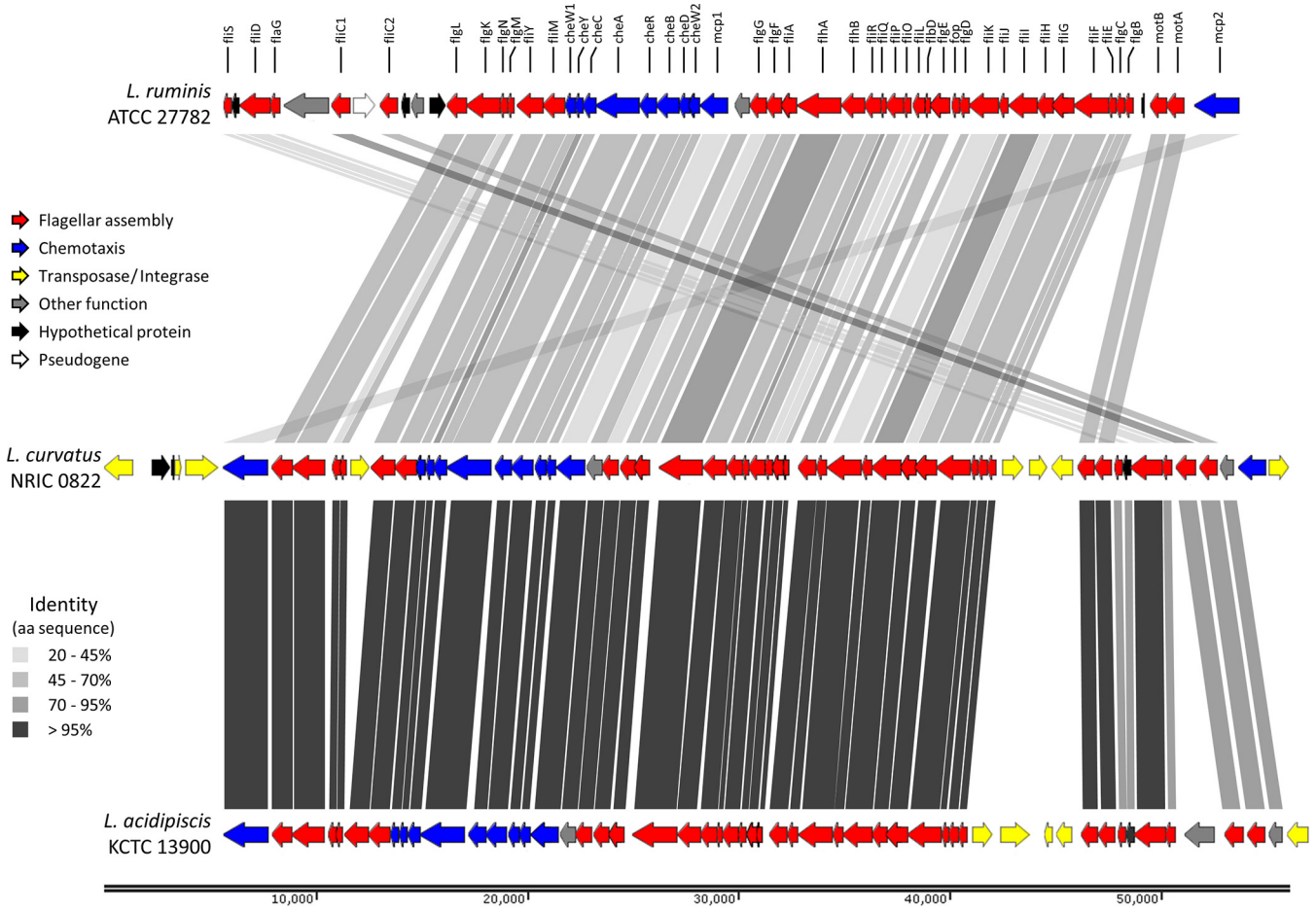

FIG 3 Comparison of motility locus organization in *L. curvatus* NRIC 0822, *L. ruminis* ATCC 27782, and *L. acidipiscis* KCTC 13900. Motility operon representations of *L. ruminis* ATCC 27782, *L. curvatus* NRIC 0822, and *L. acidipiscis* KCTC 13900 were built in SnapGene Viewer, version 2.4.3. Identity scores between protein sequences were obtained from the LALIGN Web tool by using alignment default values (see Table S4 in the supplemental material for percentages of identity).

genes and 2 potentially motile species, carrying motility genes). Examination of the motility gene composition reveals that 42 genes are present in all 16 operons (the number excludes *L. sicerae*, because it has been described too recently). This set of genes was searched for correspondence with the KEGG flagellar assembly and bacterial chemotaxis pathways (see Fig. S2 in the supplemental material). With regard to the flagellar assembly pathway, 27 of the 33 genes returned a positive hit (see Fig. S2A). All of the genes involved in the flagellum structure are present except, unsurprisingly, the L and P rings encoded by the *flgI* and *flgH* genes, specific to Gram-negative bacteria. These rings are also missing in *Bacillus subtilis* (29). The same explanation likely holds for the absence of some expression regulators (FlgA, FliT, FhlC, and FhlD) with no matches on the KEGG flagellar assembly map (see Fig. S2A). For the bacterial chemotaxis map, 13 of the 17 genes returned a positive hit; only the Aer, CheV, CheX, and CheZ proteins were not found (see Fig. S2B). Few differences in motility gene composition were observed between the 16 motility operons, and these are also in accordance with the phylogenetic analysis. Indeed, the gene encoding the flagellar protein FlaG is absent only in *L. cacaonum* DSM 21116^T and *L. mali* DSM 20444^T (at the top of the ML tree in Fig. 4B). The gene encoding the flagellar operon protein FOP is absent in the *L. acidipisicis*, *L. curvatus*, and *L. agilis* motility operons (Fig. 4B, bottom) but is present in *L. ruminis* and in all the species in the *L. mali* subclade. The gene encoding the flagellin FliC is duplicated in all the species of the *L. ruminis* subclade: *L. acidipisicis*, *L. curvatus*, *L. ruminis*, and *L. agilis* (Fig. 4B, bottom). Phylogenetic analysis of this flagellin protein, which is potentially important for interaction with the immune system via TLR5, showed a different organization than the 16S rRNA phylogeny (see Fig. S3A in the supplemental material). The two FliC proteins of *L. ruminis* ATCC 27782 and *L. agilis* DSM 20509^T were clustered together, and the *L. ruminis* proteins were clustered with those of *L. acidipiscis* and *L. curvatus*, whereas *L. agilis* FliC was clustered with the *L. vini*, *L. ghanensis*, and *L. nagelii* FliC proteins. The two FliC proteins of *L. acidipiscis* and *L. curvatus* were clustered with homologs (see Fig. S3A). Almost all the amino acids important for the interaction with the TLR5 receptor are well conserved. A few substitutions were evident and involved similar amino acids (see Fig. S3B). From an evolutionary point of view, across all 16 motility operons, the CheY protein (a chemotaxis regulator transmitting a signal to a flagellar motor component) presents the highest residue identity, at 68%, and the lowest minimum evolution number, with 0.133 ± 0.020 amino acid differ-

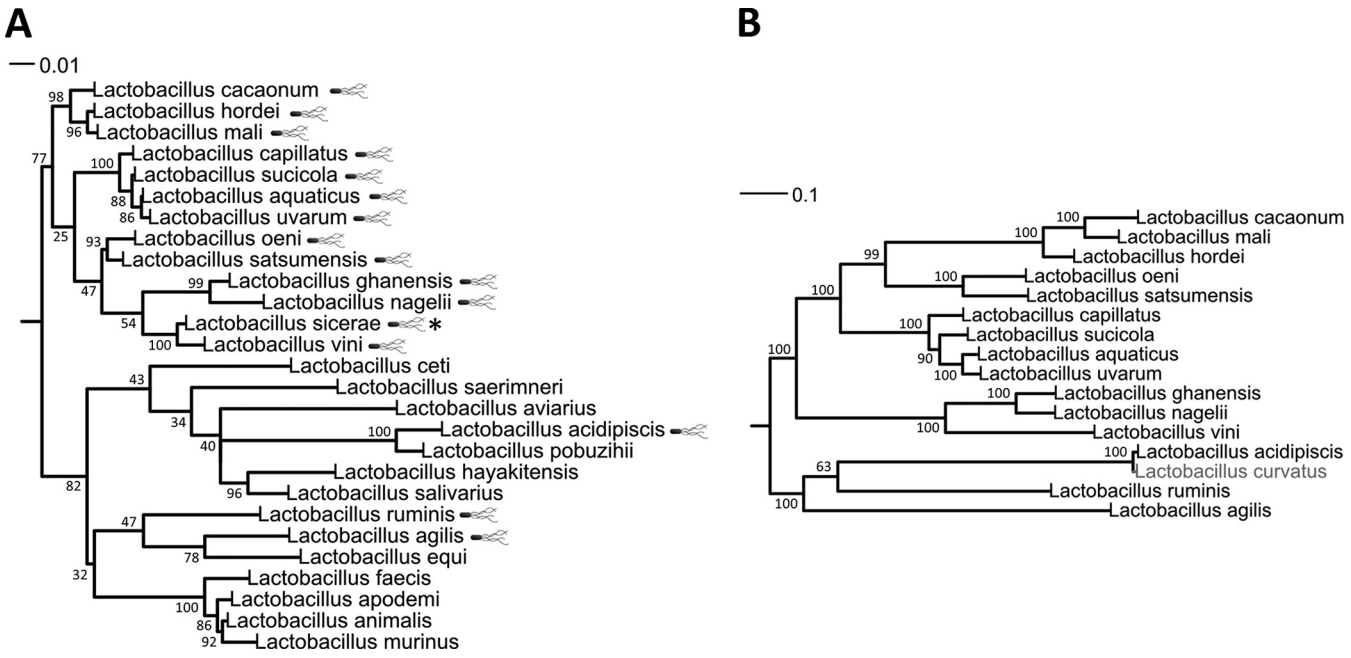

FIG 4 Concordant phylogeny of rRNA genes and motility proteins in the *L. salivarius* clade. (A) 16S rRNA gene ML subtree displaying only the *L. salivarius* clade. , motile *Lactobacillus* species; *, the motile species *L. sicerae* (11) was not included in this study because this new species was described too recently, and the partial draft genome did not allow us to identify all the motility proteins. (B) Phylogenetic analysis of motility proteins in lactobacilli. This phylogenetic tree is based on concatenated protein sequence analysis of the predicted motility genes and depicts the phylogenetic relationships among motile species of the genus *Lactobacillus*. The sequences were aligned with MUSCLE, and the trees were inferred with MEGA6 software (25). The evolutionary history was inferred by using the maximum likelihood method based on the LG model. Statistical support was estimated with bootstraps (1,000 replicates).

ence per site, from averaging over all sequence pairs (see Table S7 in the supplemental material). The most divergent proteins are FliK (1.49% identity and 0.783 amino acid difference per site) and FlaG (4.43% identity and 0.593 amino acid difference per site). Two other predicted proteins were present in all 16 operons: the cell division ATP-binding protein FtsE and a hypothetical protein that might be involved in motility. The sizes of the motility operons range from 39 kb for *L. ghanensis* DSM 18630^T and *L. nagelii* DSM 13675^T to 70 kb for *L. capillatus* DSM 19910^T. Mobile genetic elements were found in 10 of the 16 motility operons, either inside the operon or flanking it. Some of the lactobacillus motility operons have a very simple organization, harboring only motility genes, but other motility loci are interrupted by genes with no known role in motility.

DISCUSSION

The property of motility is biologically important for bacteria and may potentially confer competitive advantages, such as nutrient acquisition and niche colonization. This would justify the established metabolic costs associated with the assembly and energization of flagella (5). The number of *Lactobacillus* species that have been reported to be motile makes up only a small proportion of the 179 species that currently belong to the genus *Lactobacillus*, but that number has been increasing in recent years and merits further investigation. From an evolutionary perspective, the almost complete restriction of flagellate species to the *L. salivarius* clade is also noteworthy (5). To date, 17 *Lactobacillus* species have been historically described as motile. Origins of isolation differ greatly, from fermented food products (cheese, cider, wine, cocoa) to environmental sources (sap of oak tree, sewage, and freshwater pond) and animal sources (bovine rumen) (Table 1). This great diversity of sources for motile *Lactobacillus* species is indicative of the importance of flagellum-meditated motility and the advantages that likely accompany this trait, such as niche colonization or biofilm formation. Interestingly, the species of the *L. salivarius* clade isolated from vertebrates are not motile (except for *L. ruminis*), and the species isolated from the environment are motile (except for *L. pobuzihii*) (see Fig. S4 in the supplemental material). We hypothesize that motility genes were selected against while the lactobacillus was in contact with a host. This hypothesis seems to agree with the study of Cullender et al. (30) showing that the development of flagellin-specific adaptive immune responses can downregulate or select against the production of flagella by the gut microbiome.

This study presents unequivocal evidence for the existence of a motile strain/species outside the *L. salivarius* clade, which, until now, comprised all known motile species. These findings were supported by both phenotypic and genomic data. *L. curvatus* NRIC 0822 was nonmotile in the stationary phase, which is in accordance with a previous study on *L. ruminis* ATCC 27782 and suggests that nutrient depletion may influence the motility phenotype (5). A previous study also reported that the chemotaxis/motility operon of *L. ruminis* L5 was upregulated in the "late" growth phase (optical density at 600 nm [OD_{600}], ~1.0) in comparison to the very "early" growth phase (OD_{600}, ~0.1) (31), so the growth phase must be considered when one is deciding on motility phenotypes. Likewise, we noted three genes (in motile *Lactobacillus* species) that harbored frameshifts that should abol-

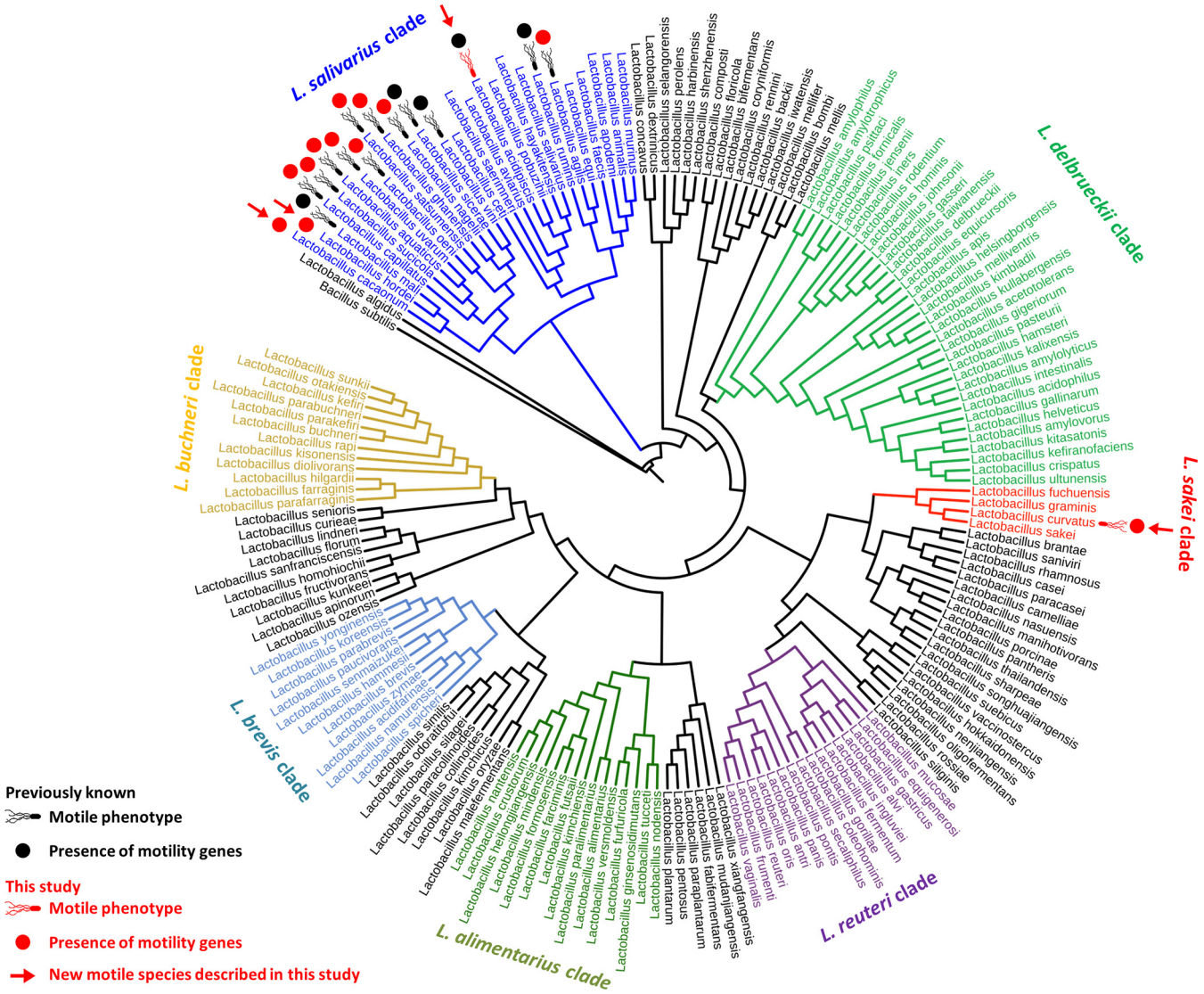

FIG 5 16S rRNA gene phylogenetic tree and motility of lactobacilli. The tree, based on 16S rRNA gene sequence analysis, depicts the phylogenetic relationships among species of the genus *Lactobacillus*. The 16S rRNA gene sequences were aligned with MUSCLE, and the tree was inferred with MEGA6 software (25). The evolutionary history was inferred by using the maximum likelihood method based on the general time-reversible model. Statistical support was estimated with bootstraps (1,000 replicates). The main clades are indicated according to the last taxonomic update of the lactobacilli (12). Motile *Lactobacillus* species (Table 1) described previously are shown in black, and species newly recognized as motile in this study are shown in red.

ish motility. Selection against motility in laboratory culture can lead to such mutations, as in the case of the *fliP* mutation in *Helicobacter pylori* strain 26695 (32). Other studies have described motile species of *Lactobacillus*, but often these studies are quite old, the strains are not available for testing, and the species-level identification of the isolates or strains in question was probably not robust (33). For example, Torriani et al. reported that some strains of *L. curvatus* are motile but lose their motility upon subculture. This motile phenotype may be unreliable because the conditions for testing were not reported, and there was no molecular investigation of motility genes (34). Another recent study from Cullender et al. described the motility of *L. brevis* DSM 20054^T and *L. sakei* NRRL B-1917, but these strains did not stimulate TLR5, and the Western blot experiment targeting the flagellin proteins did not identify a reacting protein (*L. brevis*) or displayed a protein with a molecular mass too high (≈250 kDa for *L. sakei*) for it to be a flagellin protein (the mean molecular mass of the predicted lactobacillus flagellin proteins in this study is 33.01 ± 3.72 kDa) (30). In addition, the genome of *L. brevis* DSM 20054^T is available online (and was also sequenced in the *Lactobacillus* genome-sequencing initiative), and none of the motility motifs returned significant hits in this genome during our analysis. More recently, *L. koreensis* DCY50^T was described as motile (35), but the motility agar used contained only 0.15% agar (very soft, compared to the usual 0.3 to 0.5% agar for motility tests), and the TEM or atomic force microscopy (AFM) micrographs failed to show any flagella, leading us to question the motility of this strain. *L. koreensis* strain DCY50^T was retested in our study and was not

shown to be motile, and none of the motility motifs returned significant sequence matches (data not shown). In the future, when a new *Lactobacillus* sp. is suspected to be motile, it would be desirable to test the presence of motility genes by PCR to confirm the observed motile phenotype.

TEM and light microscopy images of the motile *L. curvatus* NRIC 0822 cell showed the presence of peritrichous flagella, also observed in *L. ruminis* ATCC 27782 (5) but different from the polar flagellum observed in *L. sicerae* CECT 8227^T (11) and *L. ruminis* L5 (31). The organization and genes of the *L. curvatus* NRIC 0822 and *L. acidipiscis* KCTC 13900 motility operons are very similar (98.8% identity between the concatenated proteins), suggesting that the *L. curvatus* NRIC 0822 motility genes were horizontally acquired recently. This is strongly supported by the presence of mobile genetic elements inside and flanking the *L. curvatus* NRIC 0822 motility operon. This proposed horizontal transfer would therefore have occurred between members of two distinct *Lactobacillus* clades: *L. acidipiscis* in the *L. salivarius* clade and *L. curvatus* in the *L. sakei* clade. In addition, the motility of *L. acidipiscis* was newly described in this study. *L. acidipiscis* KCTC 13900 is motile at 30°C but not at 37°C (data not shown), which can explain the nonmotile phenotype recorded for this strain to date, since all the culture for this species was previously performed at 37°C (20, 36). In kabura-zushi, *L. acidipiscis* was not detected (37), whereas this species has been found together with *L. curvatus* in a similar fermented food, narezushi, produced in the same area of Japan (38).

The motility operon structure in *Lactobacillus* spp. seems to be relatively conserved, with identical gene blocks. This selection presumably results in efficient flagellum assembly, allowing the flagellar substructures serving as checkpoints to coordinate flagellar gene expression with assembly (39). Some of the *Lactobacillus* motility operons have a very simple organization, but other motility loci are interrupted by genes with no known role in motility, leading to the size variability observed between the lactobacillus motility operons (from 39 to 70 kb). For example, the inclusion of genes for rhamnose utilization in the motility loci of *L. mali* DSM 20444^T, *L. cacaonum* DSM 21116^T, and *L. sucicola* DSM 21376^T, and of glycosyltransferase genes in the motility loci of *L. ruminis* ATCC 22782, *L. agilis* DSM 20509^T, and *L. vini* DSM 20605^T, may indicate that the flagellin proteins of these species are modified by glycosylation, since rhamnose has been found as a posttranslational modification of flagellin in other bacteria (40).

In the future, it will be necessary to investigate the expression level of the motility operon *in vivo* in the gut and to determine whether the flagellum is assembled in this environment. A previous study suggested that some control occurs in the gut to render commensal gut bacteria generally nonmotile, explaining the low levels of flagellin protein in a healthy gut despite the capacity of the gut microbiome to produce flagella (41, 42). This control might be due to the ability of flagellin-specific IgA to inhibit bacterial motility and downregulate flagellar gene expression *in vitro* (30).

In the mammalian gut, the microbe-associated molecular patterns (MAMPs) of commensal microbiota are recognized by host pattern recognition receptors (PRRs), such as the TLRs. This interaction may contribute to homeostasis and protection from injury in the gut (43). The flagellin-TLR5 interaction leads to a proinflammatory response (44), also shown with *L. ruminis* TH14 (45) and with flagellated cells and flagellin proteins of *L. ruminis* ATCC 27782 (5). The relatively high conservation of the TLR5 interaction amino acid motif among all the motile *Lactobacillus* species suggests that all flagellin proteins in this species are able to trigger this immune response via TLR5.

This study provides global phenotypic, genetic, and phylogenetic insights into flagellum-mediated motility in lactobacilli. The characterization of the *in vivo* expression level and the immune response to flagellated lactobacilli may become particularly relevant in the context of probiotic, pharmaceutical, or vaccination applications.

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