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Distribution of Megaplasmids in *Lactobacillus salivarius* and Other Lactobacilli▽†

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The genome of *Lactobacillus salivarius* UCC118 includes a 242-kb megaplasmid, pMP118. We now show that 33 strains of *L. salivarius* isolated from humans and animals all harbor a megaplasmid, which hybridized with the *repA* and *repE* replication origin probes of pMP118. Linear megaplasmids that did not hybridize with the pMP118 *repA* probe were also found in some strains of *L. salivarius*, showing for the first time that a lactic acid bacterium has multiple megaplasmids. Phylogenetic analysis of the *repE* and *groEL* sequences of 28 *L. salivarius* strains suggested similar evolutionary paths for the chromosome and megaplasmid. Although the replication origin of circular megaplasmids in *L. salivarius* was highly conserved, genotypic and phenotypic comparisons revealed significant variation between megaplasmid-encoded traits. Furthermore, megaplasmids of sizes ranging from 120 kb to 490 kb were present in seven strains belonging to six other *Lactobacillus* species from among 91 strains and 47 species tested. The discovery of the widespread presence of megaplasmids in *L. salivarius*, and restricted carriage by other *Lactobacillus* species, provides an opportunity to study the contribution of large extrachromosomal replicons to the biology of *Lactobacillus*.

Lactic acid bacteria (LAB) are a diverse group of gram-positive bacteria that are found in a wide variety of nutrient-rich environments (29). LAB have been extensively exploited because of their ability to preserve food, beverages, and forage (43). Many LAB have been subjected to comprehensive genetic and genomic analyses (33, 40, 42) to provide a molecular basis for understanding their distinctive properties, some of which are determined extrachromosomally. Plasmids are commonly found in many members of the lactic acid bacteria (25). Many of these plasmids are cryptic (61), and their contribution to the biology of the strain harboring them is unclear. However, it is recognized that plasmid-borne traits are major accessories that are key to the phenotypes of industrially important groups such as the lactococci (reviewed in reference 45). Noteworthy lactococcal properties that are plasmid encoded include the production of the PrtP protease (12), abortive infection mechanisms to prevent bacteriophage attack (6, 49), exopolysaccharide biosynthesis (69), and bacteriocin production (66). DNA sequencing of the four plasmids harbored by *Lactococcus lactis* strain SK11, a widely used dairy strain, identified a broad repertoire of novel genes that significantly enhance or expand the metabolism, fitness, and stress resistance of the bacterium (62). The ability of plasmids to undergo dissemination by conjugation or other processes underlines their potential importance for contributing significant but variable traits to LAB.

Comprising over a hundred species, the genus *Lactobacillus* represents the largest group within the family *Lactobacillaceae* (15, 60). The lactobacilli, like LAB in general, are associated with foodstuffs, plants, and animals (27), and many species are used in industrial applications such as food production (reviewed in reference 63). Some members of this genus are also attributed with "probiotic" properties (reviewed in references 17, 18, 26, 31, 32, 57, and 58), meaning the conferring of benefits to the consumer over and above inherent nutrition (26). This has contributed to heightened interest in genomics of the lactobacilli, and 10 genome sequences from nine *Lactobacillus* species have now been determined (1, 9, 13, 34, 42, 50, 67). As for other LAB, plasmids are commonly found in lactobacilli, and multiple extrachromosomal replicons are often present in a single strain (70). The lactobacilli whose genomes were sequenced are therefore somewhat anomalous for their paucity of plasmid content. *Lactobacillus plantarum* WCFS1 and *Lactobacillus salivarius* UCC118 each harbor three plasmids (13, 34), while the sequenced strains of *Lactobacillus casei* and *Lactobacillus brevis* contain one and two plasmids, respectively (42). However, the genomes of the sequenced strains of *Lactobacillus acidophilus* (1), *Lactobacillus johnsonii* (50) and *Lactobacillus sakei* (9) and two strains of *Lactobacillus bulgaricus* (42, 67) all lack any plasmids.

The three plasmids of *L. plantarum* WCFS1 have been functionally analyzed (68). The two smaller plasmids had no annotated genes related to functions other than replication. The largest plasmid, pWCFS103 (36 kb), conferred resistance to arsenate/arsenite and was shown to be conjugative. *Lactobacillus* plasmids from nonsequenced strains/species confer properties similar to those described previously for other plasmids of LAB (reviewed in reference 70), such as carbohydrate utilization (10), bacteriocin production (48), and exopolysaccharide biosynthesis (35). Interestingly, the sequence of the plasmid genome of *Lactobacillus paracasei* strain NFBC338 identified genes related to collagen adhesion and biotin utilization (16), which may be relevant for the probiotic properties of this strain.

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The genome of *L. salivarius* strain UCC118 includes three plasmids (13), pSF20 (20 kb), pSF44 (44 kb), and pMP118 (242 kb). Plasmids pSF20 and pSF44 are almost exclusively cryptic (22), and their contribution to phenotype is currently unclear. Megaplasmid pMP118 is the largest sequenced plasmid in LAB. The annotation of pMP118 suggested that it conferred a range of additional metabolic capabilities upon *L. salivarius* UCC118, such as rhamnose and sorbitol utilization. Furthermore, it completed the genetic complement for encoding the pentose phosphate pathway (13), which allowed it to utilize ribose. pMP118 also harbored genes that are likely to contribute to host colonization or probiotic properties such as a bile salt hydrolase and the production of the broad-spectrum two-component bacteriocin Abp118 (23). Hybridization analysis of S1 nuclease-treated genomic DNA of nine other *L. salivarius* strains identified related megaplasmids in all strains.

We noted in the sequence description of pMP118 that plasmids greater than 100 kb had previously been suggested for *L. acidophilus* (48) and *Lactobacillus gasseri* (56) but that those and other analyses of *Lactobacillus* plasmid content predated pulsed-field gel electrophoresis (PFGE) and the usage of conditions to separate megaplasmids. In the present study, we examined the distribution and relatedness of megaplasmids in a large and diverse panel of *L. salivarius* strains as well as in other members of the genus.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 33 strains of *Lactobacillus salivarius* used in this study are listed in Table 1. Ninety-one strains belonging to 47 species of *Lactobacillus* used in this study are listed in Table 2. Unless otherwise indicated, lactobacilli were routinely cultured at 37°C under microaerobic conditions (5% CO_2) in de Man-Rogosa-Sharpe (MRS) medium (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom).

Chemicals. Low-melting-pointing (LMP) agarose, PFGE certified agarose, and λ DNA PFGE marker were purchased from Bio-Rad Laboratories (Hercules, CA). Sarkosyl (*N*-lauroylsarcosine), lysozyme, proteinase K, mutanolysin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO). *Aspergillus oryzae* S1 nuclease was purchased from Roche (Mannheim, Germany). All reagents were of analytical grade or high quality.

PFGE plug preparations. Agarose gel plugs of high-molecular-weight DNA for PFGE were prepared according to a protocol described previously (3), with some small modifications, as outlined below. All *Lactobacillus* strains were grown in MRS broth supplemented with 0.5 g/liter cysteine in an anaerobic jar, at a temperature specified in Table 2, to early stationary phase. A volume of culture containing approximately 10^9 bacteria (equivalent to an optical density at 600 nm of 2.0) was centrifuged (20,000 × *g* for 1 min), washed once with 1 ml NT buffer (1 M NaCl, 10 mM Tris-HCl [pH 7.6]), and repelleted (20,000 × *g* for 1 min). The cell pellet was resuspended in 450 μl NET buffer (1 M NaCl, 100 mM EDTA, 10 mM Tris-Cl [pH 7.6]). An equal volume of melted 2% (wt/vol) LMP agarose, prepared in 0.125 M EDTA (pH 7.6) and maintained at 50°C, was added. The cell suspension and LMP agarose were mixed carefully without introducing bubbles. Gel plugs were formed by pipetting 300-μl volumes into plug molds and were allowed to solidify at 4°C for 10 min. Up to three plugs per strain were added to 2 ml of NET buffer containing 1% (wt/vol) sarkosyl, 10 mg/ml lysozyme, and 40 U/ml mutanolysin and then incubated at 37°C for 24 h. The lysozyme solution was replaced with 5 ml of 0.5 M EDTA (pH 8.0) containing 1% (wt/vol) sarkosyl and 0.5 mg/ml proteinase K and then incubated at 37°C for 24 h. This step was repeated with a fresh proteinase K solution. Plugs were then washed with 5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 1 mM PMSF (freshly prepared) at 37°C for 1 h to inactivate the proteinase K. This was followed by two 30-min incubations in 5 ml TE buffer at room temperature to remove the PMSF. Plugs were stored in 10 mM Tris-HCl–100 mM EDTA (pH 8.0) at 4°C.

S1 nuclease treatment. Single slices (2 mm by 2 mm) were soaked in 200 μl S1 buffer (50 mM NaCl, 30 mM sodium acetate [pH 4.5], 5 mM $ZnSO_4$) at room temperature for 30 min. The S1 buffer was replaced with another 200 μl S1 buffer containing 1 unit of *A. oryzae* S1 nuclease and then incubated at 37°C for 45 min. The reaction was stopped by replacing the S1 buffer with 200 μl of 0.5 M EDTA (pH 8.0) and held stationary at room temperature for 10 min. The 0.5 M EDTA was replaced with 200 μl TE and left still at room temperature for at least 30 min before loading onto a gel.

TABLE 1. *L. salivarius* strains used in this study[a]

Strain	Origin	Reference(s)
UCC118	Human ileal-cecal region	13, 65
AH4231	Human ileal-cecal region	65
AH4331	Human ileal-cecal region	65
AH43310	Human ileal-cecal region	65
AH43324	Human ileal-cecal region	65
AH43348	Human ileal-cecal region	65
DSM20492	Human saliva	
DSM20554^T	Human saliva	53
DSM20555^T	Human saliva	53
NCIMB8816	Italian human saliva	
NCIMB8817	Turkey feces	
NCIMB8818	St. Ivel cheese	
NCIMB702343	Unknown	
CCUG27530B	Human abdomen, abscess	
CCUG38008	Human gall, 73-year-old male	
CCUG43299	Human blood	
CCUG45735	Human blood	
CCUG47825	Human blood, 55-year-old female	
CCUG44481	Bird	
CCUG47171	Human tooth plaque	
CCUG47826	Human blood, 55-year-old female	
JCM1040	Human intestine	46
JCM1042	Human intestine	46
JCM1044	Human intestine	46
JCM1045	Human intestine	46
JCM1046	Swine intestine	46
JCM1047	Swine intestine	46
JCM1230	Chicken intestine	46
UCC119	Chicken cecum	
01M14315	Human gallbladder pus	71
LMG14476	Cat with myocarditis	
LMG14477	Parakeet with sepsis	
L21	Human feces	45

[a] CCUG, Culture Collection University Göteborg; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; LMG, Laboratorium voor Microbiologie, Universiteit Gent; NCIMB, National Collections of Industrial Food and Marine Bacteria. Type strains are indicated by a superscript T.

PFGE. Plug slices were loaded directly into the wells of a 1% (wt/vol) PFGE agarose gel melted in 0.5× TBE (89 mM Tris-borate, 2 mM EDTA [pH 8.3]) buffer. The wells were sealed with molten 1% LMP agarose in 0.5× TBE buffer. DNA fragments were resolved using a CHEF-DR III pulsed-field system (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm for 20 h with 0.5× TBE running buffer maintained at 14°C. Linear ramped pulse times were selected depending on the size of DNA fragments to be resolved; for routine analyses, a linear ramped pulse time of 3 s to 50 s was employed. Gels were stained in distilled water containing 0.5 μg/ml ethidium bromide for 120 min under light-limited conditions.

Probe preparation and Southern hybridization. Gels were depurinated for 10 min in 0.2 M HCl, denatured for 30 min in 0.5 M NaOH–1.5 M NaCl, neutralized for 45 min in 0.5 M Tris (pH 7.5)–1.5 M NaCl, transferred by capillary overnight to Hybond-N^+ nylon membranes (Amersham Biosciences, United Kingdom), and cross-linked to the membrane with UV light. The primers used to generate PCR amplicons that were used as probes are listed in Table S1 in the supplemental material.

To detect genes present on megaplasmids from *L. salivarius* strains, membranes were probed with PCR products as detailed in Table S1 in the supplemental material. Five hundred nanograms of probe DNA was labeled with the enzyme horseradish peroxidase according to the instructions for the ECL direct nucleic acid labeling and detection kit (Amersham Biosciences, United Kingdom). Membranes were prehybridized in 20 ml ECL hybridization buffer containing 5% blocking agent and 0.3 M NaCl at 42°C for 30 min, prior to the

TABLE 2. Other *Lactobacillus* strains used in this study

Strain	Growth temp (°C)	Origin	Reference or source
L. sobrius DSM16698^T	37	Feces, piglet intestine; The Netherlands	36
L. amylovorus DSM20552	37	Intestine of adult	
L. kitasatonis DSM16761^T	37	Chicken, intestine; Japan	47
L. ultunensis DSM16047^T	37	Gastric biopsies, human stomach mucosa; Kalix, Sweden	54
L. crispatus DSM20356	37	Turkey feces	
L. acidophilus ATCC4356^T	37	Human	59
L. helveticus NCDO87	37		
L. helveticus NCDO1243	37		
L. gallinarum DSM10532^T	37	Chicken crop	
L. acetotolerans DSM20749^T	30	Fermented vinegar broth	
L. hamsteri DSM5661^T	37	Feces of hamster	
L. intestinalis DSM6629^T	37	Intestine of rat	24
L. kalixensis DSM16043^T	37	Gastric biopsies, human stomach mucosa	54
L. delbrueckii subsp. *bulgaricus* DSM20081^T	37	Bulgarian yogurt	
L. delbrueckii subsp. *lactis* DSM20073	37	Saliva	
L. johnsonii DSM20553	37	Sour milk	
L. oris DSM4864^T	37	Human saliva	20
L. antri DSM16041^T	37	Gastric biopsies, human stomach mucosa; Kalix, Sweden	54
L. reuteri DSM20016^T	37	Intestine of adult	
L. reuteri DSM17509	37	Rat gut; New Zealand	
L. reuteri DSM20015	37	Manure	
L. reuteri DSM20053	37	Human feces	
L. reuteri DSM20056	37	Rat feces	
L. reuteri NCDO1359	37		
L. ingluviei DSM15946^T	37	Pigeon, crop; Belgium	2
L. ingluviei DSM14792	42	Chicken feces; Thailand	
L. fermentum DSM20055	30	Saliva	
L. gastricus DSM16045^T	37	Gastric biopsies, human stomach mucosa; Kalix, Sweden	54
L. mucosae DSM13345^T	37	Pig small intestine; Sweden	55
L. vaccinostercus DSM20634^T	30	Cow dung	
L. paracasei subsp. *paracasei* NCDO151	30		
L. paracasei subsp. *paracasei* 43362	30	Human ileocecum	
L. paracasei subsp. *paracasei* 43332	30	Human ileocecum	
L. paracasei subsp. *paracasei* 43338	30	Human ileocecum	
L. casei DSM20011^T	30	Cheese	
L. casei NCDO1202	30	Cheese starter	
L. bifermentans DSM20003^T	30	Blown cheese	
L. pantheris DSM15945^T	37	Jaguar, feces; China	39
L. parabuchneri DSM5707^T	28	Human saliva	
L. brevis DSM20054^T	30	Feces	
L. brevis NCDO1058	30	Farmhouse red Cheshire cheese 1955	
L. malefermentans DSM5705^T	28	Beer	
L. mindensis DSM14500^T	30	Sourdough; Germany	19
L. plantarum NCDO326	30	Isolated from dental carries	
L. plantarum NCDO340	30	Isolated from silage	
L. plantarum NCDO704	30	Isolated from starter	
L. plantarum 14-E10	30	Sauerkraut	41
L. plantarum 14-F3	30	Sauerkraut	41
L. plantarum 22-E2	30	Sauerkraut	41
L. plantarum NCIMB8826	30	Human saliva	
L. pentosus DSM20314^T	30		
L. paraplantarum 14-H4	30		41
L. gramini DSM20719^T	30	Grass silage	
L. curvatus NCDO2739	30	Isolated from milk	
L. curvatus UCC7017	30		
L. sakei DSM20100	30	Stool of breast-fed infant	
L. sakei DSM6333	30	Pork, vacuum packaged; produces bacteriocin sakacin A	
L. sakei UCC7012	30		
L. sakei UCC7016	30		
L. sakei NCDO2714	30	Isolated from "moto," starter for sake	
L. sakei LMG2313	30		
L. aviarius subsp. *araffinosus* DSM20653^T	37	Intestine of chicken	

Continued on following page

TABLE 2—*Continued*

Strain	Growth temp (°C)	Origin	Reference or source
L. aviarius subsp. *aviarius* DSM20655^T	37	Feces of chicken	
L. cypricasei DSM15353^T	37	Cheese, Halloumi; Cyprus	
L. acidipiscis DSM15836^T	30	Fermented fish; Thailand	
L. equi DSM15833^T	37	Feces of horses; Japan	
L. agilis DSM20509^T	37	Municipal sewage	
L. murinus DSM20452^T	37	Intestine of rat	
L. animalis DSM20602^T	37	Dental plaque of baboon	
L. saerimneri DSM16049^T	37	Pig feces; Sweden	
L. ruminis ATCC 25644	37	Bovine rumen	
L. ruminis ATCC 27780^T	37	Bovine rumen	
L. ruminis ATCC 27781	37	Bovine rumen	
L. ruminis ATCC 27782	37	Bovine rumen	
L. ruminis L5	37	Human feces	64
L. ruminis Subject 21	37		G. W. Tannock
L. ruminis Subject 23	37		G. W. Tannock
L. ruminis Subject 36	37		G. W. Tannock
L. ruminis Subject 38	37		G. W. Tannock
L. gasseri SR21	37	Human gastric biopsy	K. A. Ryan et al., unpublished data
L. gasseri SR23	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR26	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR27	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR29	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR210	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR211	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR212	37	Human gastric biopsy	Ryan et al., unpublished
L. gasseri SR214	37	Human gastric biopsy	Ryan et al., unpublished
L. vaginalis SR28	37	Human gastric biopsy	Ryan et al., unpublished
L. vaginalis SR213	37	Human gastric biopsy	Ryan et al., unpublished
L. fermentum SR22	37	Human gastric biopsy	Ryan et al., unpublished

ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; NCDO, National Collection of Dairy Organisms; NCIMB, National Collection of Industrial and Marine Bacteria. Type strains are indicated by a superscript T.

addition of the labeled probe. Hybridization was performed at 42°C for 16 h. Membranes were washed three times for 20 min in 6 M urea–0.4% sodium dodecyl sulfate–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0) at 42°C and three times for 5 min in 2× SSC at room temperature. Autoradiographs were produced by exposing Hyperfilm ECL for 1 h to 16 h at room temperature.

Stripping the membrane and rehybridization. A previously hybridized membrane was rinsed thoroughly in double-distilled water. The membrane was washed three times for 30 min in 0.2 M NaOH containing 0.1% sodium dodecyl sulfate at 37°C to remove the bound probe. The membrane was further washed for 5 min in 2× SSC and stored in 2× SSC before hybridization with a second probe.

DNA sequencing. The sequences of LSL_1740 (*repE*) from each of the 28 *L. salivarius* strains were generated by sequencing a 2.0-kb PCR amplicon amplified by using primers 1739_F1 and 1740_R1 (see Table S1 in the supplemental material). Sequencing was performed by MWG Biotech (Ebersberg, Germany). In addition, the fidelity of all probes used for Southern hybridization was confirmed by DNA sequencing.

Phylogenetic analysis. Two different phylogenetic trees corresponding to chromosomal *groEL*- and *repA*-type megaplasmid *repE* genes for 28 *L. salivarius* strains were analyzed. Maximum likelihood trees were built using the best model (TrN+I for *groEL* and TIM+I for *repE*) with the Web-based tool MULTIPHYL (30) with a bootstrap value of 100. Gap regions were excised manually. The *groEL* sequences used in this study were reported in our previous study (38).

Carbohydrate utilization assay. The ability of various *L. salivarius* strains to ferment ribose, sorbitol, and rhamnose was tested using API 50 CH strips in conjunction with API 50 CHL medium (bioMerieux). Details of this method were described in a previous study (38).

Bacteriocin assay. *L. salivarius* strains were grown on MRS plates at appropriate temperatures for 16 h and then flooded with MRS sloppy agar (MRS broth plus 0.75% agar) containing *Listeria monocytogenes* EGDe as an indicator, with *L. salivarius* UCC118 as a positive control. Colonies that produced a halo against *L. monocytogenes* EGDe were recorded as being bacteriocin-positive strains.

Nucleotide sequence accession numbers. The sequences of the *repE* gene from 28 *L. salivarius* strains were deposited in GenBank under accession numbers EF452504 to EF452531 (specified by strain in Fig. 4).

RESULTS

The presence of megaplasmids is a general feature of *L. salivarius*. Ten *L. salivarius* strains were previously shown to harbor a megaplasmid whose size ranged from 100 kb to 380 kb (13). We therefore hypothesized that the presence of megaplasmids might be a general feature of *L. salivarius*. To test this, we collected another 23 *L. salivarius* strains from a variety of origins, including gastrointestinal tract isolates and clinical strains (Table 1). The presence of megaplasmids in these 23 strains was analyzed by the S1 PFGE protocol combined with Southern analysis that was described in our previous study (13).

A survey of the additional 23 strains revealed that all strains harbor a megaplasmid that hybridized to the LSL_1739 (*repA*) probe derived from the sequenced megaplasmid pMP118 from *L. salivarius* strain UCC118 (Fig. 1). We therefore designated the megaplasmids in the 23 strains used in this study, and the 10 strains described in our previous study (13), as *repA*-type megaplasmids. We also noted the variable presence of DNA bands migrating close to the respective *repA*-type megaplasmids, e.g., in strains UCC118, AH43310, CCUG27530B, CCUG45735, CCUG44481, and CCUG47826 (Fig. 1). By Southern hybridization, we established that the apparently 195-kb band in the

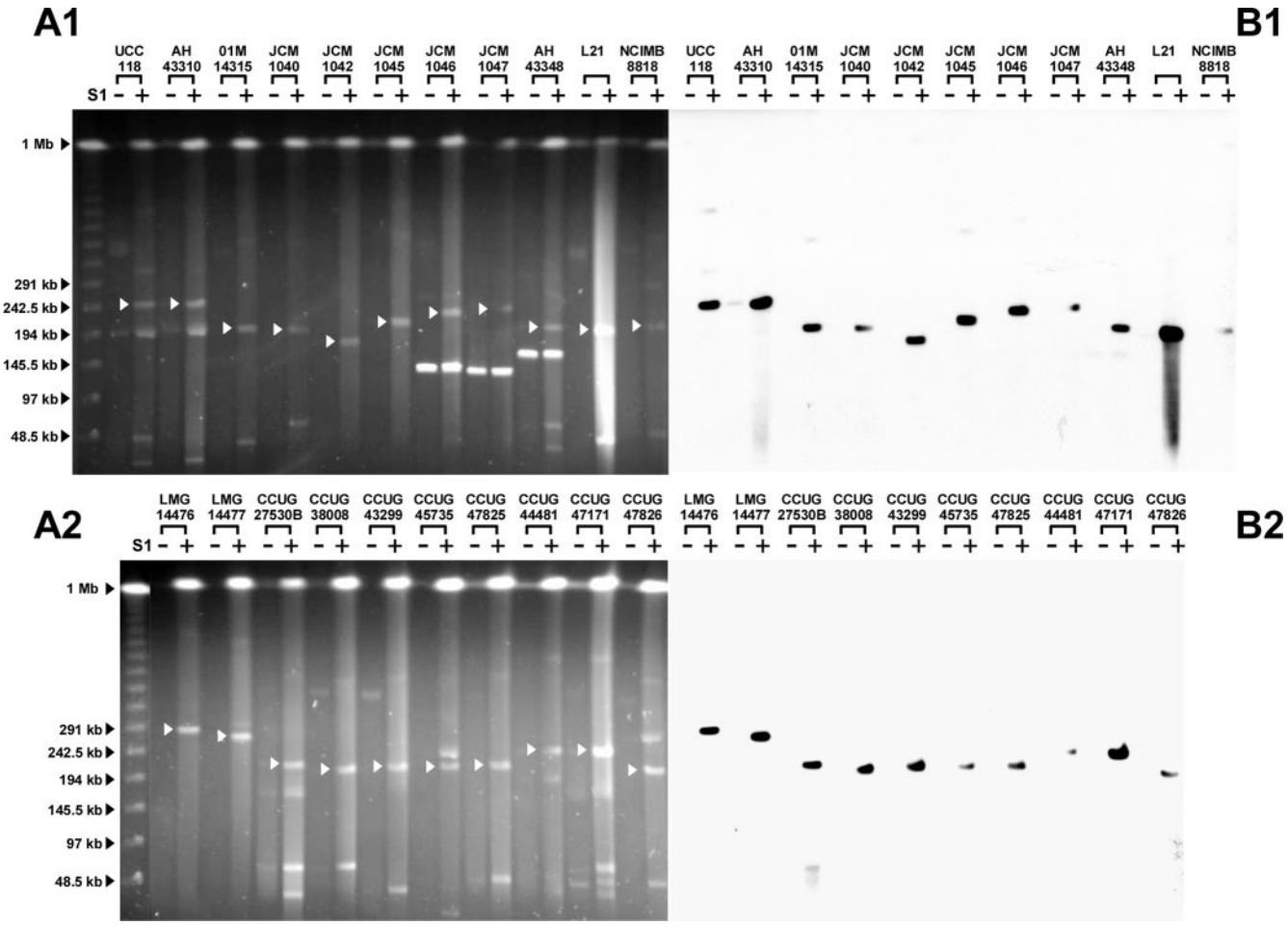

FIG. 1. *repA*-type megaplasmids are widely present in *L. salivarius*. (A1 and A2) PFGE of genomic DNA of 23 *L. salivarius* strains with strain UCC118 as a positive control. (B1 and B2) Corresponding Southern hybridization with the pMP118 *repA* probe. + and − indicate presence and absence of treatment with S1 nuclease, respectively. PFGE was run at 6 V/cm at 14°C for 20 h using a linear ramped pulse time of 3 s to 50 s. Black arrows indicate DNA size standards. White arrows indicate the S1 nuclease-linearized megaplasmid band that hybridized with the pMP118 *repA* probe. The prominent smear in the lane for strain L21 indicates genomic DNA degradation due to overtreatment with S1 nuclease.

UCC118 lane was actually another form of the 20-kb plasmid pSF20 in strain UCC118 (data not shown). This suggests that the supercoiled or open circular forms of small plasmids migrate much slower than the corresponding linear form in PFGE. The presence of small plasmids may therefore complicate establishing if a given strain has megaplasmids, presenting a technical obstacle in surveying the presence of megaplasmids. To circumvent this, all genomic DNA plugs of *L. salivarius* strains were studied by multiple PFGE experiments under various running conditions. The linearized DNA fragment would thus be expected to migrate to the same position relative to linear DNA markers, while supercoiled or open circular forms of a plasmid should display inconsistent migration relative to linear DNA markers. A comparison between Fig. 1 and Fig. 2A exemplifies the behavior of *repA*-type megaplasmids, which migrate to a constant position relative to the linear DNA marker, while the other bands close to the corresponding megaplasmids migrate differently under two running conditions. This suggests that strains UCC118, AH43310, CCUG 45735, and CCUG47826 each harbor only one circular megaplasmid of the *repA* type. Similar data were obtained for strains CCUG27530B and CCUG44481 (data not shown).

Apart from the universal presence of the *repA*-type megaplasmids in *L. salivarius*, S1 PFGE revealed that genomic DNA of *L. salivarius* strains JCM1046, JCM1047, and AH43348 included a prominent band whose running behavior was not affected by S1 nuclease treatment. This is indicative of the topology expected for a linear megaplasmid. Changing the PFGE running conditions further confirmed that strains JCM1046, JCM1047 (data not shown), and AH43348 harbor linear megaplasmids (Fig. 2B). To our knowledge, this is the first time that a lactic acid bacterium is reported to contain multiple megaplasmids. For clarity and consistency of nomenclature, we designated the *L. salivarius* circular *repA*-type megaplasmids pMP followed by the strain name, while the linear megaplasmid was designated pLMP followed by the strain name. None of the linear megaplasmids in strains JCM1046, JCM1047, and AH43348 hybridized with the *repA* probe, showing that the replication of these linear megaplasmids is different from that of the *repA*-type circular megaplasmids, However, the linear megaplasmid pLMP43348 in strain AH43348 had a sequence that hybridized to the probe for the presumptive chromosome partitioning ATPase (*parA* [LSL_1741]), while pLMP1046 and pLMP1047 did not (Fig. 3). Because the 33

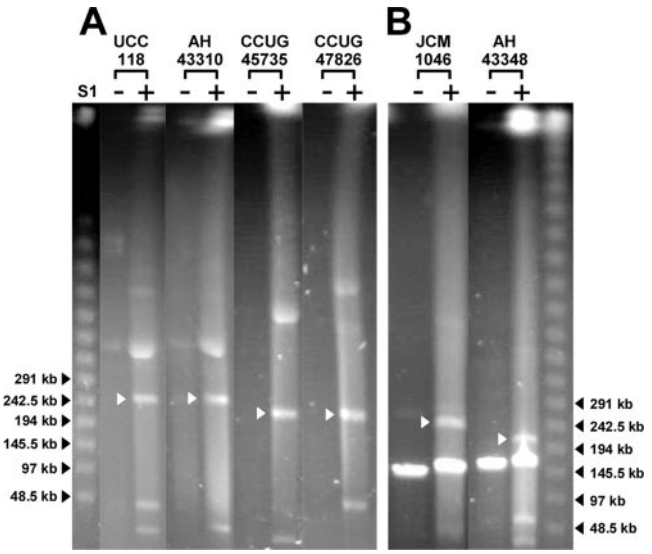

FIG. 2. Confirmation of the presence of circular and linear megaplasmids in selected strains. + and − indicate the presence and absence of treatment with S1 nuclease, respectively. PFGE was run at 6 V/cm at 14°C for 20 h using a linear ramped pulse time of 30 s to 60 s. (A) Confirmation of the presence of a single circular megaplasmid in *L. salivarius* strains (refer also to the migration patterns in Fig. 1). (B) Confirmation of the coexistence of a circular megaplasmid and a linear megaplasmid in *L. salivarius* strains. White arrows indicate linearized *repA*-type circular megaplasmids. A and B were grouped from different parts of the same gel.

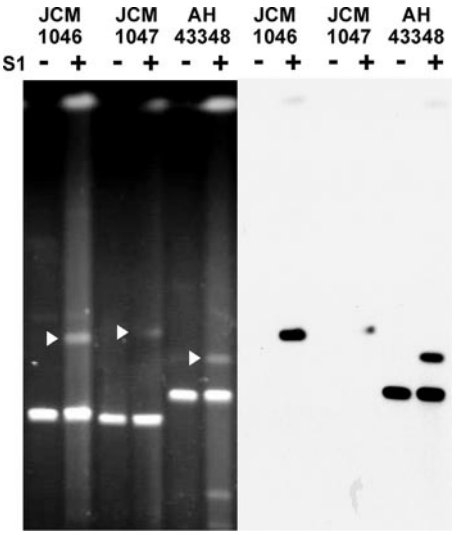

FIG. 3. pLMP43348 in strain AH43348 contains sequences that hybridize to the *parA* probe of pMP118. + and − indicate the presence and absence of treatment with S1 nuclease, respectively. PFGE was run at 6 V/cm at 14°C for 20 h using a linear ramped pulse time of 3 s to 50 s. White arrows indicate linearized *repA*-type circular megaplasmids. The gel pattern in A is the same as that shown in Fig. 1, while that in B was generated by stripping the membrane used in Fig. 1 and rehybridizing it with the *parA* probe.

strains that we tested all contain at least one *repA*-type megaplasmid, we conclude that the presence of megaplasmids is a general genomic feature of this species.

Phylogenetic analysis of *repA*-type megaplasmids in *L. salivarius*. Further Southern analysis revealed that all *L. salivarius* *repA*-type megaplasmids also hybridized with the LSL_1740 (*repE*) and LSL_1741 (*parA*) probes (Table 3), which are located downstream of the *repA* gene. Primers spanning the *repA* and *repE* genes amplified a similarly sized amplicon in 28 *L. salivarius* strains tested (data not shown). This suggests that all *L. salivarius* *repA*-type megaplasmids have a highly conserved replication backbone. In order to compare the evolutionary histories of chromosomes and megaplasmids in *L. salivarius*, the *groEL* and *repE* genes from the respective replicons were subjected to molecular phylogeny. The *groEL* gene has been extensively used as an alternative molecular marker in phylogenetic analyses since it is a highly conserved housekeeping gene (38). *repE* was selected as a megaplasmid molecular marker, as it is universally present, and sequencing of the 2-kb *repAE* amplicon revealed that *repE* is more divergent than *repA* (data not shown). This analysis was performed for 28 of the 33 *L. salivarius* strains, since PFGE analysis of chromosomal digests identified two pairs of presumptively identical strains (AH43310-AH43324 and AH4331-AH4231), which were previously shown to have near-identical phenotypic profiles (38). In addition, PCRs for the *repA-repE* amplicon failed for strains DSM20554 and CCUG38008, presumably due to a 3′ mismatch, since they were shown by hybridization to harbor the *repA* and *repE* genes on their respective megaplasmids. Finally, strain AH43348 was a late addition to the strain panel and was not used for *repE* gene phylogeny. Maximum likelihood trees were constructed for the *groEL* and *repE* genes of the 28 strains (Fig. 4). Both trees clearly show two major branches although with a different level of confidence, as indicated by the bootstrap values. Although not totally concordant, there was a strong agreement between trees constructed based upon *repE* of the megaplasmid and the corresponding chromosomal *groEL* gene of the host strain. Longer tree branch lengths indicated by a higher relative scale in the *repE* tree are the consequence of a higher mutation rate of this gene than that of *groEL*. Interestingly, sequences from human-derived strains clustered better with each other than with those of animal origin, which is particularly evident in the lower branch. Moreover, despite the approximately 2:1 ratio (18/10) of human isolates to animal isolates, it is apparent that when the *repE* tree is analyzed, the major branches more consistently represent animal-derived strains for the upper branch and human-derived strains for the lower branch.

Biological and genetic features encoded on the *L. salivarius* *repA*-type megaplasmids. DNA sequencing (13) identified contingency metabolism genes on pMP118, which were predicted to increase the metabolic flexibility of the host bacterium. The ability to assimilate sorbitol, rhamnose, and ribose by strain UCC118 was experimentally confirmed, verifying observations at the genomic level (38). As the replication backbone of the *repA*-type megaplasmids is highly conserved, we next investigated the degree of conservation of other genetic or phenotypic features on the other *repA*-type megaplasmids. The positions of the probes used to investigate the genetic features on the pMP118 map are shown in Fig. S1 in the supplemental material.

Sorbitol-fermenting ability was considered to be a universal feature for all strains in the original description of *L. salivarius* (53) and in the current API50 biochemical test profile. However,

TABLE 3. Distribution of genes related to replication, sugar metabolism, and bacteriocin production on the *repA*-type megaplasmids in 33 strains of *L. salivarius*[a]

repA-type megaplasmid	Strain	Size of megaplasmid (kb)	LSL_1739 (*repA*)	LSL_1740 (*repE*)	LSL_1741 (*parA*)	Sorbitol utilization		Rhamnose utilization		Ribose utilization			Bacteriocin production	
						Phenotype	LSL_1894	Phenotype	LSL_1752 (*rhaB*)	Phenotype	LSL_1888 (*mipB*)	LSL_1946 (*tktA*)	Phenotype	LSL_1916 + LSL_1917 (*Abp118*α + *Abp118*β)
pMP20555	DSM20555	380[b]	+	+	+	F	+	F	+	**NF**	**+**	**+**	*NBA*	*N*
pMP14476	LMG14476	290	+	+	+	F	+	F	+	**NF**	**+**	**+**	NBA	+
pMP14477	LMG14477	270	+	+	+	F	+	F	+	**NF**	**+**	**+**	NBA	+
pMP20554	DSM20554	260[b]	+	+	+	F	+	*NF*	*N*	**NF**	**+**	**+**	*NBA*	*N*
pMP118	UCC118	242[b]	+	+	+	F	+	F	+	F	+	+	BA	+
pMP43310	AH43310	240	+	+	+	F	+	F	+	F	+	+	BA	+
pMP43324	AH43324	240	+	+	+	F	+	F	+	F	+	+	BA	+
pMP20492	DSM20492	240[b]	+	+	+	F	+	WF	+	**NF**	**+**	**+**	*NBA*	*N*
pMP44481	CCUG44481	240	+	+	+	F	+	*NF*	*N*	NF	+	N	**NBA**	**+**
pMP1047	JCM1047	240	+	+	+	F	+	*NF*	*N*	NF	+	N	**NBA**	**+**
pMP47171	CCUG47171	240	+	+	+	F	+	F	+	**NF**	**+**	**+**	**NBA**	**+**
pMP1046	JCM1046	230	+	+	+	F	+	F	+	**NF**	**+**	**+**	**NBA**	**+**
pMP1045	JCM1045	220	+	+	+	F	+	F	+	**NF**	**+**	**+**	**NBA**	**+**
pMP47825	CCUG47825	220	+	+	+	F	+	F	+	**WF**	**+**	**+**	**NBA**	**+**
pMP45735	CCUG45735	220	+	+	+	F	+	F	+	**WF**	**+**	**+**	**NBA**	**+**
pMP27530B	CCUG27530B	220	+	+	+	F	+	**NF**	**+**	**WF**	**+**	**+**	**NBA**	**+**
pMP47826	CCUG47826	220	+	+	+	F	+	F	+	**NF**	**+**	**+**	**NBA**	**+**
pMP43299	CCUG43299	218	+	+	+	F	+	*NF*	*N*	WF	+	+	*NBA*	*N*
pMP38008	CCUG38008	215	+	+	+	F	+	*NF*	*N*	*WF*	+	*N*	**NBA**	**+**
pMP4231	AH4231	210[b]	+	+	+	F	+	**NF**	**+**	**NF**	**+**	**+**	BA	+
pMP4331	AH4331	210	+	+	+	F	+	**NF**	**+**	**NF**	**+**	**+**	BA	+
pMP14315	01M14315	200	+	+	+	F	+	*NF*	*N*	**NF**	**+**	**+**	*NBA*	*N*
pMP119	UCC119	195[b]	+	+	+	*NF*	*N*	F	+	NF	N	N	BA	+
pMP43348	AH43348	195	+	+	+	F	+	**NF**	**+**	WF	+	+	ND	+
pMP8818	NCIMB8818	195	+	+	+	F	+	WF	+	**NF**	**+**	**+**	**NBA**	**+**
pMP1040	JCM1040	195	+	+	+	*NF*	*N*	*NF*	*N*	**NF**	**+**	**+**	*NBA*	*N*
pMP21	L21	190	+	+	+	F	+	F	+	**NF**	**+**	**+**	**NBA**	**+**
pMP8816	NCIMB8816	180[b]	+	+	+	F	+	*NF*	*N*	NF	+	N	*NBA*	*N*
pMP1042	JCM1042	180	+	+	+	F	+	*NF*	*N*	NF	+	N	*NBA*	*N*
pMP1044	JCM1044	180	+	+	+	F	+	*NF*	*N*	NF	+	N	*NBA*	*N*
pMP702343	NCIMB702343	160[b]	+	+	+	*NF*	*N*	WF	+	NF	N	N	**NBA**	**+**
pMP8817	NCIMB8817	145[b]	+	+	+	*NF*	*N*	**NF**	**+**	NF	N	+	*NBA*	*N*
pMP1230	JCM1230	100[b]	+	+	+	F	+	*NF*	*N*	**NF**	**+**	**+**	**NBA**	**+**

[a] +, gene detected by hybridization; N, no hybridization signal detected; F, carbon source fermented; WF, carbon source weakly fermented; NF, carbon source not fermented; BA, bacteriocin activity detected; NBA, no bacteriocin activity detected; ND, not determined. Boldface type indicates that the gene is present but has a conflicting phenotype; italic type indicates that the gene is absent and has a corresponding phenotype. Data for carbohydrate-fermenting tests were obtained from our previous study (38). Indicator strains used in this study for determining the bacteriocin activity were *L. monocytogenes* EGDe and *L. sake* LMG2313.

[b] The size of these *repA*-type megaplasmids were estimated in a previous study (13).

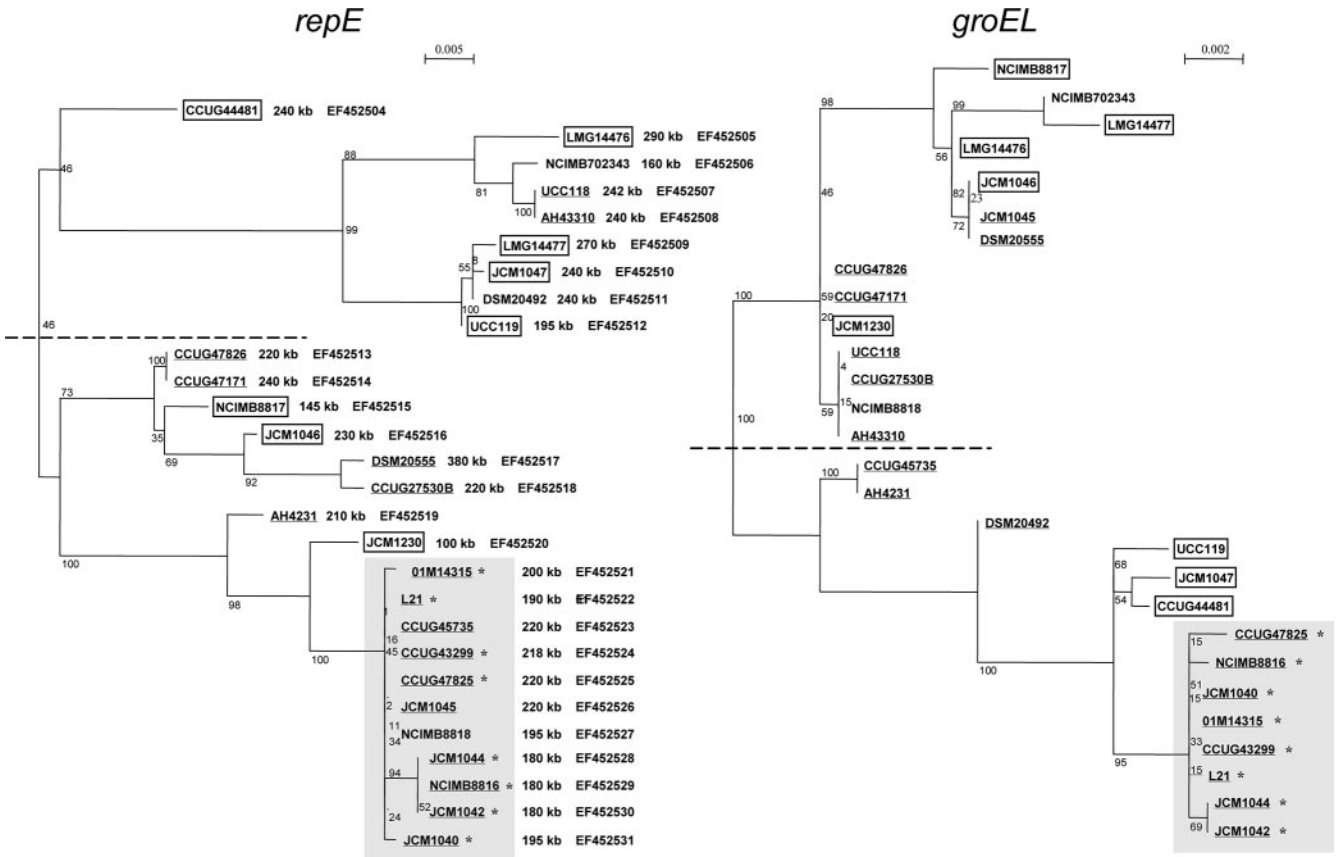

FIG. 4. Phylogenetic analysis of the *L. salivarius repE* genes of the *repA*-type megaplasmids and *groEL* gene phylogeny of the same strains. The size of the individual *repA*-type circular megaplasmids is shown to the right of the respective strain name in the *repE* tree. GenBank accession numbers for *repE* sequences are shown to the right of the megaplasmid sizes. Boxed and underlined strain labels represent animal origin and human origin, respectively. Gray shading indicates clusters that are relatively conserved between trees; strains located in this area in both phylogenies are labeled with an asterisk.

4 strains out of 33 tested were unable to ferment sorbitol (Table 3). Interestingly, all 29 strains that fermented sorbitol harbored an LSL_1894 gene homolog encoding sorbitol-6-phosphate 2-dehydrogenase on their respective *repA*-type megaplasmids, as revealed by Southern hybridization (Table 3), while in the four strains that did not ferment sorbitol, this gene appears to be absent.

Historically, a rhamnose-fermenting ability was considered to be a criterion to distinguish *L. salivarius* subsp. *salivarius* from *L. salivarius* subsp. *salicinius* (53). We unified these two subspecies into a single species in a previous study, since not all *L. salivarius* subsp. *salivarius* strains can ferment rhamnose, while some *L. salivarius* subsp. *salicinius* strains can also ferment rhamnose (38). The rhamnose fermentation pathway in UCC118 involves LSL_1752 (*rhaB* [rhamnulokinase]), LSL_1754 (L-rhamnose isomerase), and LSL_1755 (*araD* [rhamnulose-1-phosphate aldolase]). As shown in Table 3, 11 strains that do not have an LSL_1752 homolog failed to ferment rhamnose. In addition, there are five strains that appear to possess an LSL_1752 homolog but that do not ferment rhamnose, suggesting gene silencing or a lack of other genes that are essential for completing the pathway.

L. salivarius was considered to be homofermentative when it was described in 1953 (53). This was corrected in our previous study (13), as pMP118 encodes transketolase and transaldolase, which complete the pentose phosphate pathway. Despite this, the ability to ferment pentose, e.g., ribose, is very rare among *L. salivarius* strains (38). Using LSL_1888 (encoding transaldolase) and LSL_1946 (encoding transketolase) as probes, we found that these two homologs are actually widely present on the *repA*-type megaplasmids (Table 3). Nearly half of the strains have sequences that hybridize with the LSL_1888 and LSL_1946 probes but lack the ability to ferment ribose.

The production of the bacteriocin Abp118 is megaplasmid encoded, and bacteriocin production might be a competitive advantage for commensal organisms. It may also be a useful trait for bacterial strains used as probiotic ingredients. Significantly, we have recently shown that Abp118 production is the major mechanism whereby *L. salivarius* UCC118 dramatically reduces *Listeria monocytogenes* infection in a mouse model (14). Sequences hybridizing to a probe for the Abp118 locus were detected in 20 strains. However, only 6 out of these 20 isolates produce detectable levels of bacteriocin against *L. monocytogenes* EGDe (Table 3).

Presence of megaplasmids in other lactobacilli. The discovery of megaplasmids in all *L. salivarius* strains examined prompted us to investigate the presence of megaplasmids across the genus *Lactobacillus*. We collected 91 strains belonging to 47

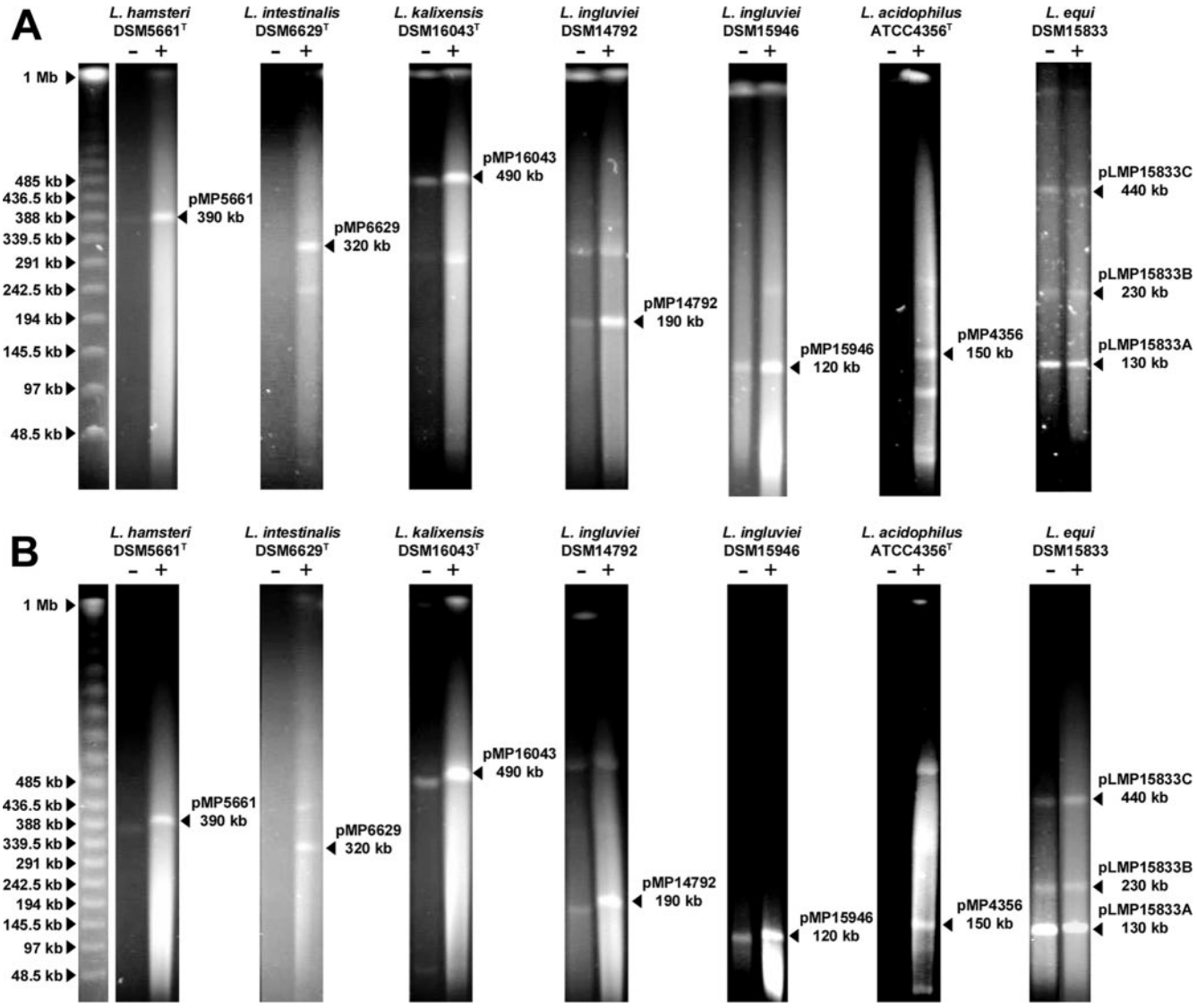

FIG. 5. Megaplasmids of various sizes are found in a restricted number of other lactobacilli. Genomic DNA of seven strains belonging to six different *Lactobacillus* species with (+) or without (−) S1 nuclease treatment was resolved by PFGE at 6 V/cm at 14°C for 20 h using a linear ramped pulse time of 3 s to 50 s (A) or from 30 s to 60 s (B). Arrowheads to the left indicate size standards. Brightly smeared material below the pMP15946 band (120 kb) from *L. ingluviei* DSM15946 represents degraded genomic DNA. Arrowheads to the right of individual photos indicate the sizes of the megaplasmids confirmed by two different PFGE running conditions. Originally, the PFGE of *L. equi* DSM15833 was run in the loading order of S1 +/−, but we cropped it from the original gel picture and regrouped it in an order of S1 −/+ for consistency with other strains.

species (excluding *L. salivarius*) and screened them for the presence of megaplasmids by the S1 PFGE protocol. The phylogenetic positions of the species investigated are shown in Fig. S2 in the supplemental material. Megaplasmids of sizes ranging from 120 kb to 490 kb were detected in 7 strains belonging to 6 species from among the 91 strains and 47 species examined. These seven strains are as follows: *Lactobacillus hamsteri* DSM5661, *L. intestinalis* DSM6629, *L. kalixensis* DSM16043, *L. ingluviei* DSM14792, *L. ingluviei* DSM15946, *L. acidophilus* ATCC 4356, and *L. equi* DSM15833. Figure 5 shows the PFGE patterns for megaplasmid-containing strains run under two different conditions as described above. All megaplasmid-containing strains represent isolates from the gastrointestinal tract. These megaplasmids failed to hybridize with the pMP118 *repA* probe, suggesting that the *L. salivarius* megaplasmids represent a unique megaplasmid group in lactobacilli. *L. equi* DSM15833 harbored a prominent linear megaplasmid band and two other potential megaplasmids. Within the species that are phylogenetically most closely related to *L. salivarius*, only one of nine species (*L. equi*) was shown to have megaplasmids, which were not related to the *repA*-type megaplasmids. This suggests that the distribution of the *Lactobacillus* megaplasmids is independent of the phylogeny of the genus.

DISCUSSION

The occurrence of megaplasmids in lactic acid bacteria was not widely recognized until pMP118 was identified in *L. sali-*

varius by genome sequencing (13). At that time, we identified circular megaplasmids in nine other *L. salivarius* strains. We previously defined pMP118 as a megaplasmid because it contains neither tRNA nor rRNA genes; it has plasmid-related replication and partition proteins, and it does not contain the only copy of any known essential gene in the genome (13). In the present study, *L. salivarius* megaplasmids that have not been sequenced are functionally defined as those greater than 100 kb in size, by which we confirm the carriage of megaplasmids in 33 strains from very diverse sources. Plasmid pMP118, at 242 kb, constitutes 11% of the *L. salivarius* genome. The megaplasmid of strain DSM20555 (390 kb) could constitute 17.8% of the genome, assuming that its chromosome is around 1.8 Mb. Among the 33 *repA*-type circular megaplasmids identified in *L. salivarius* in this study, the average size was 218 ± 47 kb, with 22 *repA*-type circular megaplasmids larger than 200 kb. The contribution of a substantial proportion of the *L. salivarius* genome by a single extrachromosomal replicon is probably a universal phenomenon for this species.

The identification of megaplasmid molecules is facilitated by the S1 nuclease PFGE protocol (3). Definitive megaplasmid identification, particularly in strains that harbor one or more smaller circular plasmids, typically requires multiple PFGE runs under various conditions. This may have confounded the identification of large plasmids in LAB in previous studies. Multiple PFGE runs also contributed robustness to our identification of linear megaplasmids in three *L. salivarius* strains. The existence of a linear megaplasmid in a *Lactobacillus* strain was previously indicated by one report on *L. gasseri* strain CNRZ222 (56), which was in the same size range (150 kb) as the linear megaplasmids in *L. salivarius* (140 kb, 145 kb, and 175 kb) reported herein. Linear plasmids are well characterized in prokaryotes such as *Streptomyces coelicolor* (4), *Borrelia burgdorferi* (21), and *Escherichia coli* (51). The linear plasmid N15 in *E. coli* is actually the lysogenic form of a lambdoid phage (52), and linear prophages in *Klebsiella oxytoca* (8) and *Yersinia enterocolitica* (28) have also been described. However, these linear plasmid-prophage replicons are considerably smaller than the linear *Lactobacillus* megaplasmids reported here. It will be interesting to examine the organization of *Lactobacillus* linear megaplasmids, particularly the mode of replication, and the traits that they carry. Interestingly, the hybridization of LSL_1741 (*parA*) to pLMP43348 suggested that the partitioning of this linear megaplasmid is dependent upon a protein that is similar to that employed by circular megaplasmids. A similar conclusion was reported previously for the *Mycobacterium celatum* 23-kb linear plasmid pCLP, which has a genetic locus similar to the maintenance genes (*par* operon) of a bacterial circular plasmid (37). The pCLP *par* operon was shown to be important for the stability of this linear plasmid.

All circular megaplasmids in *L. salivarius* carried *repA* and *repE* gene homologs, and the *repE* gene showed sufficient divergence to allow phylogenetic analyses. It is conceivable that large plasmids such as those analyzed here might have multiple origins and that the resulting lack of selective pressure on the *repE* genes would make them unsuitable for phylogeny. However, the *repA-repE* locus is the only identifiable replication region in pMP118. The *repE* genes sequenced from the other plasmids are also linked to a *repA* homolog, and both *rep* genes are very similar to the corresponding genes of pMP118. Finally, none of the *repE* sequence variations used for phylogeny disrupts the reading frame. Since the pMP118 *repE* gene is functional, it is highly likely that the *repE* genes used for phylogeny are also functional. Although concordance was not absolute, the general agreement between trees based upon the *repE* and *groEL* genes of the respective strains suggests that the acquisition of a megaplasmid was a relatively early event in the evolution of *L. salivarius*.

The sorbitol utilization locus was well conserved, with only four of the strains that were analyzed appearing to have lost the corresponding gene from their respective megaplasmids and with the phenotype being consistent with the genotype in all cases. However, almost half the strains tested lacked the rhamnulokinase gene LSL_1752. Conversely, many strains that were unable to ferment ribose harbored both the transaldolase and transketolase genes on their respective megaplasmids. A total of eight strains lacked either or both of these genes. It is plausible that migration from the chromosome to the megaplasmid of part of the genetic information for the pentose phosphate pathway has been followed by the decay of relevant coding sequences in either replicon in particular strains. This makes it more remarkable that a minority of the strains examined have retained the functionality of the pathway.

The bacteriocin Abp118 has broad-spectrum activity (23) that would be expected to contribute to competitive exclusion and strain competitiveness of the producing strain in the gastrointestinal tract. Despite this, exactly one-third of the *L. salivarius* strains tested failed to hybridize with the *abp118* gene probe, and the origin of this trait is unclear. It is also currently unknown if a loss of sequences or gene function, e.g., of induction mechanisms or ancillary genes, is responsible for the lack of bacteriocin production in the 14 out of 20 strains that harbor the genes for the Abp118 peptides.

In addition to *L. salivarius*, we detected the presence of megaplasmids in 6 other species from among 47 tested across the whole phylogenetic range of this very diverse genus (7). Four of these species are members of group A of the 16S rRNA phylogeny (7) that includes the so-called *L. acidophilus* complex: *L. acidophilus* (human gastrointestinal tract), *L. hamsteri* (hamster feces), *L. intestinalis* (rat intestine), and *L. kalixensis* (human stomach mucosa). *L. ingluviei*, two strains of which harbor megaplasmids, was isolated from pigeon crop, and *L. equi* was isolated from equine feces. Thus, all of these species are found in the gastrointestinal tract, and while this correlation must be treated with caution, it is clear that megaplasmids are uncommon in extraintestinal lactobacilli, food-associated lactobacilli, and free-living species. The lack of a homolog of the pMP118 *repA* gene establishes them as genetically distinct, at least at replication level, from the *L. salivarius* pMP118 replicon. *L. equi* DSM15833 apparently harbors three linear megaplasmids, representing the most complex genome geometry noted in this study. We note, however, that the intensities of the larger two plasmid bands is significantly lower than those of other linear megaplasmids in this strain and in *L salivarius* strains. These bands could therefore represent other forms of the linear plasmid pLMP15833A, although they do migrate consistently as linear bands under different switching conditions.

The megaplasmid of *L. kalixensis* strain DSM16043, at 490

kb, represents the largest plasmid identified in this study and may be considered a minichromosome depending on whether or not it carries essential genes. Recent sequencing projects have uncovered a complicated array of possible bacterial genome architectures (reviewed in reference 5), and the largest bacterial genome sequenced to date (*Rhodococcus* sp. strain RHA1) (44) includes three linear megaplasmids of 332 kb, 442 kb, and 1.1 Mb. It has been suggested that linear plasmids arose by the recombination of plasmids with bacteriophages, and linear chromosomes arose by the recombination of linear plasmids with circular chromosomes (11). The complexity of genomes such as *Rhodococcus* sp. strain RHA1 illustrates the possible outcomes of such processes, which may be ongoing in *L. salivarius* and other lactobacillus species. Further characterization of circular and linear megaplasmids of lactobacilli at the sequence level will elucidate biological traits that may have been selected during megaplasmid evolution and may possibly identify source organisms for the lateral transfer of linear plasmids.

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