Removal of Antibiotic Resistance Gene-Carrying Plasmids from *Lactobacillus reuteri* ATCC 55730 and Characterization of the Resulting Daughter Strain, *L. reuteri* DSM 17938[∇]

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The spread of antibiotic resistance in pathogens is primarily a consequence of the indiscriminate use of antibiotics, but there is concern that food-borne lactic acid bacteria may act as reservoirs of antibiotic resistance genes when distributed in large doses to the gastrointestinal tract. *Lactobacillus reuteri* ATCC 55730 is a commercially available probiotic strain which has been found to harbor potentially transferable resistance genes. The aims of this study were to define the location and nature of β-lactam, tetracycline, and lincosamide resistance determinants and, if they were found to be acquired, attempt to remove them from the strain by methods that do not genetically modify the organism before subsequently testing whether the probiotic characteristics were retained. No known β-lactam resistance genes was found, but penicillin-binding proteins from ATCC 55730, two additional resistant strains, and three sensitive strains of *L. reuteri* were sequenced and comparatively analyzed. The β-lactam resistance in ATCC 55730 is probably caused by a number of alterations in the corresponding genes and can be regarded as not transferable. The strain was found to harbor two plasmids carrying *tet*(W) tetracycline and *lnu*(A) lincosamide resistance genes, respectively. A new daughter strain, *L. reuteri* DSM 17938, was derived from ATCC 55730 by removal of the two plasmids, and it was shown to have lost the resistances associated with them. Direct comparison of the parent and daughter strains for a series of in vitro properties and in a human clinical trial confirmed the retained probiotic properties of the daughter strain.

The use of probiotic bacteria, generally members of the genera *Lactobacillus* and *Bifidobacterium*, in foods and supplements that promote health or prevent disease, has followed a long history of safe use of these organisms (8). The spread of antibiotic resistance determinants in the environment is undoubtedly primarily a consequence of indiscriminate use of antibiotics in both animals and humans. However, this concern has led to the idea that food-borne lactic acid bacteria may act as reservoirs of antibiotic resistance genes when purposely distributed in large doses to the microbiota of the gastrointestinal tract (17, 38). The outcome of the EU PROSAFE project was to recommend that all future probiotics should not contain known antibiotic resistance traits (40), and currently the European Food Safety Authority qualified presumption of safety proposals seem to be following this recommendation (6).

Bacterial antibiotic resistance can be either intrinsic or acquired (16). Intrinsic resistance is a natural resistance present in all strains of a bacterial species, while acquired resistance is often identifiable as a resistance found in only a certain number of members of a particular species. Although intrinsic resistance is generally accepted to be nontransferable, acquired resistance may be more easily transferred to other bacterial species, particularly if the resistance trait is located on a plasmid in the carrier strain (37).

Lactobacillus reuteri ATCC 55730, a commercially available, well-documented probiotic (health-promoting) bacterium (1, 23, 27, 34, 46–48), has been shown to possess a series of intrinsic antibiotic resistances common to this particular species (18, 25, 26). It does, however, carry specific, unusual resistances to tetracycline and lincosamides, as well as a β-lactam resistance which appears in approximately half of the members of this species (18, 24). With a draft genome sequence of *L. reuteri* ATCC 55730 determined in our laboratory (7), the aim of this study was to define the origin, localization, and character of these unusual resistances. Subsequent localization of *tet*(W) and *lnu*(A) on plasmids led to the removal of these plasmids and validation of the properties retained by the daughter strain generated.

MATERIALS AND METHODS

Bacteria, culturing, DNA preparation, and primers. The bacterial strains and primers used in this study are listed in Tables 1 and 2, respectively. The primers were obtained from Invitrogen (Carlsbad, CA). *L. reuteri* strains were grown in MRS broth or on MRS agar (Oxoid, Basingstoke, United Kingdom). Plates were incubated in an anaerobic atmosphere (GasPak; BD, Sparks, MD). For antibiotic supplementation, 100 µg ml^{-1} tetracycline (Sigma, St. Louis, MO; MRS-Tet) or 8 µg ml^{-1} lincomycin (Sigma; MRS-Lin) was used unless otherwise stated. Bacterial DNA was prepared with the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Identification of plasmids and putative resistance genes. Identification of plasmids in *L. reuteri* ATCC 55730 was achieved by analysis of the draft genome sequence of this strain (7). The genes encoding putative penicillin resistance proteins and penicillin-binding proteins (Pbp) were identified by BLAST searches (3) against the genome sequence with known resistance determinants (GenBank accession numbers: Bla1, AAK53749; Bla2, AAS42360; BlaZ,

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TABLE 1. *L. reuteri* strains used in this study

Strain	Description	Origin or reference
ATCC 55730	Alternative name, SD2112; Tet^r Lin^r Amp^r; human milk isolate	Biogaia AB
ATCC 55730Tet^s	ATCC 55730 cured of plasmid pLR581; Tet^s Lin^r Amp^r	This study
DSM 17938	ATCC 55730 cured of plasmids pLR581 and pLR585; Tet^s Lin^s Amp^r	This study
DSM 20016	Type strain; Tet^s Lin^s Amp^s; human intestinal isolate	DSMZ[a]
DSM 20015	Amp^s; cow manure isolate	DSMZ
ATCC 55148	Amp^s; chicken intestinal isolate	Biogaia AB
ATCC 55149	Amp^r; turkey intestinal isolate	Biogaia AB
CF48-3A	Amp^r; human fecal isolate	Biogaia AB

[a] DSMZ, German Collection of Microorganisms and Cell Cultures.

CAB94802; FibA, CAB89120; FibB, CAB89121; MecA, CAG39068; MecR1, CAG39069; MurM, NP_664650; MurN, AAK07416) and Pbp (Pbp1a, NP_720910; Pbp1b, NP_722289; Pbp2a, NP_722252; Pbp2b, NP_721030; Pbp2x, NP_720898; PbpX, NP_721297). Also, the tetracycline and lincomycin resistance genes *tet*(W) and *lnu*(A) were searched for by using known resistance genes (GenBank accession numbers ABC18266 and YP_473355, respectively).

Sequencing of Pbp-encoding genes. For all identified Pbp-encoding genes, one primer pair located close to the start and end of the gene was designed and for all but lr1752, one pair located approximately 500 bp from the first pair was also designed (Table 2). The primers were used for PCR amplification of the genes encoding the Pbp from ampicillin-sensitive *L. reuteri* strains DSM 20016, DSM 20015, and ATCC 55148 and ampicillin-resistant strains ATCC 55730, ATCC 55149, and CF48-3A. illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala, Sweden), primers (10 pmol of each), and DNA (0.5 μl) were added to the PCR mixture and amplified with the following program: 95°C for 5 min; 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min; and 72°C for 10 min. The PCR products were purified and thereafter sequenced with the same set of primers. After assembly of the genes (with Contig Express [Invitrogen]), the corresponding protein sequences were aligned and compared with *Lactoba-* *cillus* Pbp obtained from genomes available in GenBank (*L. acidophilus*, CP000033; *L. brevis*, CP00041; *L. casei*, CP000423; *L. delbrueckii*, CR954253; *L. gasseri*, CP000413; *L. helveticus*, CP000517; *L. johnsonii*, AE017198; *L. planta-rum*, AL935263; *L. sakei*, CR936503; *L. salivarius*, CP000233).

Curing of plasmids. Plasmid curing was done by protoplast formation and regeneration essentially as described by Vescovo et al. (43). An overnight culture of ATCC 55730 was diluted to an optical density at 600 nm (OD$_{600}$) of 0.1 in a 10-ml MRS culture and grown at 37°C to an OD$_{600}$ of 0.7 to 0.8. Cells were collected by centrifugation at 3,000 × g for 10 min, washed in 10 ml Nanopure water, recentrifuged, and resuspended in 2 ml of protoplast buffer (0.2 M sodium phosphate, 0.5 M sucrose, 20 mM MgCl$_2$, pH 7.0). The cells were then mixed with an equal volume of protoplast buffer containing 10 mg ml^{-1} lysozyme (Sigma) and incubated at 37°C for 1 h. Protoplasts were harvested by centrifugation at 3,000 × g for 15 min, washed with 20 ml protoplast buffer, recentrifuged, and resuspended in 1 ml of protoplast buffer. Dilutions in protoplast buffer were then plated on MRS agar with 0.5 M sucrose for regeneration. Dilutions in Nanopure water were plated on MRS agar to assess the number of remaining whole cells. The number of CFU was determined after 1 and 2 days of anaerobic incubation at 37°C. Regenerated colonies were picked to MRS and

TABLE 2. Primers used in this study

Primer	Use	Sequence
1989f	Detection of lr1989[a] on pLR580	5′-ATT TTC CAC CCG CAT ATT CA-3′
1989r	Detection of lr1989[a] on pLR580	5′-TGC ATC ACG AAT CAA ACC AT-3′
2004f	Detection of lr2004 on pLR581	5′-AGG TGA AGC ATT TCG AGC AT-3′
2004r	Detection of lr2004 on pLR581	5′-GGC TTT CCG TCA TCA TCA GT-3′
2084f	Detection of lr2084[b] on pLR584	5′-TTT GGC TGG CAA AAT CAT TC-3′
2084r	Detection of lr2084[b] on pLR584	5′-TTT TTG CAG CAT TGA AAA CG-3′
2102f	Detection of lr2102 on pLR585	5′-GAA CGG AAG CAA CAA CGA AT-3′
2102r	Detection of lr2102 on pLR585	5′-CGT TTG GTT GGA GAA GTG GT-3′
1996f	Detection of *tet*(W)	5′-TTC GCT GGG ATA CTT GAA CC-3′
1996r	Detection of *tet*(W)	5′-TTT TTA CCT GGA CCG TTT CG-3′
2105f	Detection of *lnu*(A)	5′-TGG AAA ACA ACA AAG AGA ACA ACA CA-3′
2105r	Detection of *lnu*(A	5′-CCA GAA TGA AAA AGA AGT TGA GC-3′
545(1)f	Sequencing of pbp1a	5′-ATC AGT CAA CGC GTA GTG AGC-3′
545(1)r	Sequencing of pbp1a	5′-GCA TCG TCC CAA TCA GAT G-3′
545(2)f	Sequencing of pbp1a	5′-AAC GTA AAG CCC AAG AAG CA-3′
545(2)r	Sequencing of pbp1a	5′-TGC AGC AGA AAC TTG AGT G-3′
889(1)f	Sequencing of pbp2b	5′-CAC GTA CCA AAA AGC GTC AA-3′
889(1)r	Sequencing of pbp2b	5′-AAC CCT TGG GAC TCT GAA CA-3′
889(2)f	Sequencing of pbp2b	5′-GCA GCA ATG AGT GGA GCA TA-3′
889(2)r	Sequencing of pbp2b	5′-GTT TCA CCA GGC AAG TCG AT-3′
1005(1)f	Sequencing of pbp2a	5′-TCA GAT TTG AAA GAG CGA ATC A-3′
1005(1)r	Sequencing of pbp2a	5′-ATC GAA CAT TGC TCC ACC AT-3′
1005(2)f	Sequencing of pbp2a	5′-GCG GTT GAG GTA GAA AAC CA-3′
1005(2)r	Sequencing of pbp2a	5′-CAA AGA CTG CAT AGG CAC GA-3′
1631(1)f	Sequencing of pbp2x	5′-AGC CAC AAC GAA CAA CGA AAA AT-3′
1631(1)r	Sequencing of pbp2x	5′-TTG AAC GGT TAA AGT TGT TCC A-3′
1631(2)f	Sequencing of pbp2x	5′-AAG GCG AAT TTG CTT CTC AA-3′
1631(2)r	Sequencing of pbp2x	5′-CAG TTG TGG TCT TTC AGG CA-3′
1752f	Sequencing of pbpX	5′-TTT TTG ACG CTT TGT TCC TTT-3′
1752r	Sequencing of pbpX	5′-AAT TAT GGA GAA TAT CAT CCG AAG C-3′

[a] GenBank accession number EU620430.
[b] GenBank accession number EU620435.

MRS-Tet agar for identification of bacteria cured of pLR581 and to MRS and MRS-Lin agar for bacteria cured of pLR585. Plasmid-cured candidates were identified by nongrowth on MRS agar supplemented with antibiotics.

PCR detection of plasmids and resistance genes and repetitive-sequence-based PCR (rep-PCR) typing. The plasmids and resistance genes of strain ATCC 55730 were detected by PCR by the same method as for Pbp-encoding genes. Primers 1989f and 1989r, 2004f and 2004r, 2084f and 2084r, and 2102f and 2102r (Table 2), detecting replication protein genes on pLR580, pLR581, pLR584, and pLR585, respectively (10 pmol of each), and primers 1996f and 1996r and primers 2105f and 2105r, detecting tet(W) and lnu(A), respectively, were used.

Bacterial isolates were fingerprinted by rep-PCR. PuReTaq Ready-To-Go PCR Beads, DNA (0.5 μl), and primer 5′-GTG GTG GTG GTG GTG-3′ (20 pmol) were mixed, and the reaction was performed according to the program described by Versalovic et al. (41): 95°C for 7 min; 30 cycles of 90°C for 30 s, 95°C for 1 min, 40°C for 1 min, and 65°C for 8 min; and 65°C for 16 min. The PCR products were separated by electrophoresis (1% agarose gel in 0.5× TBE buffer) and stained with ethidium bromide. Digitalized images were captured under UV light transillumination.

Determination of MICs. $L.$ $reuteri$ strains were grown in MRS broth for 16 h at 37°C. After dilution to 10^5 CFU ml^{-1}, 1 μl of each strain was spotted onto MRS plates containing lincomycin or clindamycin (Sigma) at concentrations of 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 μg ml^{-1}. After adsorption of the drops, the plates were incubated anaerobically at 37°C for 24 h. The MIC was defined as the lowest antibiotic concentration at which there was no visible bacterial growth. MICs of tetracycline were determined by Etest (AB Biodisk) on bacteria growing on MRS agar plates according to the instructions from the manufacturer.

Colony and cell morphology. $L.$ $reuteri$ strains were grown for 48 h on MRS plates at 37°C in an anaerobic atmosphere before photographs of single colonies were taken. The morphology of the bacterial cells was investigated by phase-contrast microscopy (×400 magnification).

Fermentation pattern and reuterin production. Fermentation patterns were determined with api 50 CHL (BioMérieux, Marcy l'Etoile, France) according to the instructions from the manufacturer. Reuterin production (13) was measured by the reaction between reuterin (3-hydroxy-propinoaldehyde) produced by the $L.$ $reuteri$ strains with 2,4-dinitrophenylhydrazine to form a red hydrazone. The bacteria were grown for 48 h on MRS plates (inoculated as streaks). The plates were then overlaid with 500 mM glycerol agar (1% agar) and incubated at 37°C for 30 min. Reuterin was detected by the addition of 5 ml 2,4-dinitrophenylhy-drazine (0.1% in 2 M HCl). After 3 min incubation, the solution was poured off and 5 ml 5 M KOH was added. Red zones around the colonies demonstrated the presence of reuterin and the extent of its production.

Growth. Growth was determined by inoculation of an overnight culture to an OD$_{600}$ of 0.1 in MRS broth in tubes prewarmed to 37°C. The tubes were incubated at 37°C, and samples were taken for 8 h and the OD$_{600}$ was measured. Each strain was tested in triplicate.

Binding to mucus. Binding to ovine small intestinal mucus was performed according to Roos et al. (31). All bacterial strains were analyzed in triplicate.

Acid tolerance. Testing of survival at pH 2 was performed according to Wall et al. (45) with a synthetic stomach juice described by Cotter et al. (15) that was modified by adding no enzymes. Duplicate samples were taken on each of two occasions. Statistical significance of differences was analyzed with Student's t test.

Bile tolerance. Strains were grown for 16 h in MRS broth at 37°C, and the stationary-phase suspensions were diluted in phosphate-buffered saline to approximately 10^3 to 10^6 CFU ml^{-1}. Ten microliters of each dilution was dropped onto MRS plates containing concentrations of bovine bile of up to 6% (Sigma B3883). The plates were incubated for 72 h at 37°C in an anaerobic atmosphere, after which the colonies were counted. Bacteria in exponential phase (OD$_{600}$ of 0.5) were also tested in the same manner. Each bacterium was analyzed on two separate occasions.

Pathogen inhibition. Test strains were grown in MRS broth for 16 h and plated on MRS agar. The plates were incubated anaerobically (with Anaerogen [Oxoid]) for 24 h at 37°C. Isolated single colonies were then streaked in the center of fresh MRS agar plates and incubated anaerobically for 36 h at 37°C. The target pathogens were $Candida$ $albicans$ ATCC 28956, $Enterobacter$ $sakazakii$ ATCC 51329, $Salmonella$ $enterica$ serovar Typhimurium ATCC 14028, and $Clostridium$ $difficile$ ATCC 43593.

$C.$ $albicans$ and $E.$ $sakazakii$ were grown in 10 ml of tryptic soy broth (BBL, BD) plus 1.5% Lab Lemco (Oxoid) and incubated for 48 h at 37°C ($Candida$) or 32°C ($Enterobacter$). The pathogens were then inoculated into freshly prepared sterile tryptic soy broth plus 1.5% Lab Lemco and 1.5% agar with or without the addition of glycerol (150 mM) to a target concentration of 10^5 CFU ml^{-1} ($Candida$) or 10^6 CFU ml^{-1} ($Enterobacter$). The pathogen preparations (10 ml) were overlaid on the streaked test strains growing on the MRS plates. Plates were

incubated anaerobically at 37°C ($Candida$) or 36°C ($Enterobacter$) for 48 h. $S.$ $enterica$ serovar Typhimurium and $Clostridium$ $difficile$ were grown in 10 ml of brain heart infusion broth (BBL, BD) and reinforced clostridial medium (BD), respectively, and incubated at 37°C for 16 h. Each pathogen was then inoculated in brain heart infusion agar or reinforced clostridial medium plus 1.5% agar (40°C) with or without the addition of glycerol (150 mM) to get a concentration of 10^5 CFU ml^{-1}. Ten milliliters of this preparation was overlaid on the test strains growing on the MRS plates. Plates were incubated anaerobically at 37°C for 48 h. Inhibition halos were measured in triplicate samples on three different experimental occasions for each strain and pathogen.

Gastrointestinal passage and safety in humans. Sixteen subjects (10 female) aged 27 ± 7 (mean ± standard deviation) years were recruited after written informed consent was obtained at the Gastroenterology Unit, Department of Internal Medicine, Lund University Hospital, Lund, Sweden. The Scientific Ethical Committee of Lund University Hospital approved this study prior to its start. Inclusion criteria were an age of 18 to 65 years, written informed consent, stated availability throughout the study period, a lack of any major illnesses (allergy symptoms were acceptable), and the mental ability to understand and fulfill the protocol. Exclusion criteria were significant disease, pregnancy, use of oral antibiotics in the 2 weeks prior to ingestion of the study product, and participation in other clinical trials. Subjects were randomly assigned to receive a placebo (four subjects), an ATCC 55730 standard dose (8×10^8 CFU/day; three subjects), a DSM 17938 standard dose (8×10^8 CFU/day; four subjects), or a DSM 17938 high dose (6.5×10^{10} CFU/day; five subjects). The study was double blind with neither the subjects nor the principal investigator aware of the contents of the study product in each group.

Subjects were then asked to take a study product each day for 28 days. The study product (in sealed one-dose sachets) consisted of freeze-dried powder of the strain mixed with 1.5 g maltodextrin. The placebo contained only maltodextrin and was identical in appearance to the active products but had no detectable bacteria. Sachets were kept refrigerated at all times up to the point of consumption, and $L.$ $reuteri$ levels remained stable in parallel stability testing (data not shown). The subjects were instructed in writing to open the sachet immediately before consumption, pour the powder into a glass containing cold water (at least 100 ml), stir well for 15 s, and swallow directly.

Fecal samples (≥5 g) were collected at baseline and on days 7, 14, and 28 and also on days 42 and 56 (after a 2- to 4-week washout period). The samples were stored for maximally 1 h at room temperature and maximally 1 day in the refrigerator (not frozen) until they could be collected and delivered to the laboratory, where they were frozen (−20°C) upon arrival. For analysis of $L.$ $reuteri$ content, samples were thawed, weighed, diluted in MRS broth (1:10), and mixed thoroughly. Aliquots (1 ml) were serially diluted and spread on MRS-vancomycin agar plates (50 μg ml^{-1} vancomycin) and incubated anaerobically at 37°C for 48 to 72 h. $L.$ $reuteri$ colonies were confirmed by analysis of reuterin production with the overlay assay described above. Overlay-positive clones were picked from copied plates and analyzed for specific plasmid content to identify them as $L.$ $reuteri$ ATCC 55730-like or $L.$ $reuteri$ DSM 17938 strains.

Fasting blood samples were collected at baseline and after 28 days and analyzed by standard procedures at the Clinical Chemistry Laboratory of Lund University Hospital for Fe, total iron-binding capacity, hemoglobin, erythrocyte particle concentration (erythrocytes), blood corpuscle volume, white blood cell count, blood differential cell counts, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, albumin, glucose, calcium, sodium, potassium, phosphorus, total bilirubin, alanine aminotransferase, aspartate aminotransferase, glutamyl transferase, creatinine, urea, urate, and C-reactive protein, and even in the absence of overt fever or illness, a general bacterial analysis was done to detect the possible translocation of any bacteria into the blood (only on day 28 after last study product dose was taken).

RESULTS

Identification of plasmids and resistance genes. Analysis of the genome sequence of ampicillin-resistant strain $L.$ $reuteri$ ATCC 55730 identified no known β-lactam resistance gene, but five genes encoding putative Pbp were found (Table 3). The corresponding genes from two other penicillin-resistant and three penicillin-sensitive $L.$ $reuteri$ strains were PCR amplified and sequenced for comparison. Alignment of the deduced protein sequences showed few differences, but there were amino acid differences at four positions that correlated

TABLE 3. GenBank accession numbers of Pbp compared in this study

Strain	GenBank accession no.				
	Pbp1a	Pbp2b	Pbp2a	Pbp2x	PbpX
ATCC 55730[a,d]	DQ074831	DQ074857	DQ074867	DQ074915	DQ074930
ATCC 55149[b,d]	EU604291	EU604292	EU604293	EU604294	EU604295
CF48-3A[b,d]	EU604286	EU604287	EU604288	EU604289	EU604290
DSM 20015[b]	EU604281	EU604282	EU604283	EU604284	EU604285
ATCC 55148[b]	EU604276	EU604277	EU604278	EU604279	EU604280
DSM 20016[c]	ABQ83182	ABQ83468	ABQ83526	ABQ82854	ABQ84156

[a] Genes described in reference 7.
[b] Sequenced in this study.
[c] Sequences obtained from GenBank accession number CP000705.
[d] Penicillin resistant.

with the expression of penicillin resistance. Two of those were located in Pbp1a, where aspartic acid (Asp, D) in the sensitive strains was changed to valine (Val, V) at position 399 of the resistant strains (Asp399Val, exchange 1 [E1]) and glutamine (Gln, Q) was changed to leucine (Leu, L) at position 479 (Gln479Leu, E2), as shown in Fig. 1. E3 was located in Pbp2a, where phenylalanine (Phe, F) was changed to valine (Val, V) at position 147 (Phe147Val), and the last exchange, E4, was located in Pbp2x, where alanine (Ala, A) was changed to threonine (Thr, T) at position 526 (Ala526Thr). Comparisons with Pbp from 10 other *Lactobacillus* species showed that both E1 and E4 are located in conserved regions (Fig. 1). E1 leads to the substitution of the fully conserved aspartic acid (charged) to valine, which is an amino acid with fundamentally different properties (hydrophobic). In the case of E4, the alanine present in the sensitive strains has properties similar to those of glycine (small), which is present in all other lactobacilli, whereas the threonine present in the resistant strains has other properties (contains a hydroxyl group). Although E2 and E3 are not located in conserved regions, these substitutions also produce changed properties. E1 to E4 can be explained by point mutations of the corresponding genes. E1 is caused by a change of a GAT codon to GTT; E2, CAA to CTA; E3, TTC to GTC; and E4, GCA to ACA. The Pbp2a- and PbpX-encod-

ing genes did not show any differences that correlated with the resistance patterns of the strains examined.

The genome sequence of *L. reuteri* ATCC 55730 showed four contigs that were found to harbor plasmid-related genes. Further, these contigs were built up by an elevated number of sequencing runs, indicating a higher copy number than genes located on the chromosome. The contigs could be circularized and therefore concluded to be plasmids, and the sizes were found to be 8.1 (pLR580), 12.2 (pLR581), 14.2 (pLR585), and 19.1 (pLR584) kb. The genome of ATCC 55730 was also scanned for the *tet*(W) and *lnu*(A) genes, which have previously been detected in this strain (24), and they were found as open reading frames lr1996, located on plasmid pLR581, and lr2105, located on plasmid pLR585, respectively (Table 4).

Removal of plasmids pLR581 and pLR585 from *L. reuteri* ATCC 55730. An attempt to cure *L. reuteri* ATCC 55730 of plasmids pLR581, harboring *tet*(W), and pLR585, harboring *lnu*(A), was made. Different methods were tested (data not shown), and a method by which the bacteria first were subjected to protoplast formation with subsequent cell wall regeneration was found to be effective in removing the plasmids from the bacteria. In a first trial, pLR581 was the target. After incubation in the protoplast buffer, 100-fold more colonies were obtained on MRS plates with sucrose than on those

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Pbp1a           E1                        E2
         395           407     473           487
Sensitive  KPLMDYGPAIEYL------LGISQKQPYTLQNGI
Resistant  KPLMVFGPAIEYL------LGISQKLPYTLQNGI

Consensus  KPVLDYGPAIEYL------LGVNVPSNSGLSNAI
           **..*..*** **      .*            .*

Pbp2a         E3              Pbp2x          E4
       140         153              518        531
Sensitive  KGLGRAGFLLVKNK   Sensitive  GYRIAGKTATAQIG
Resistant  KGLGRAGVLLVKNK   Resistant  GYRIAGKTTTAQIG

Consensus  KGIARA----AINK   Consensus  GYRIAVKTGTAQIA
           *. *.                        . **.*.*.
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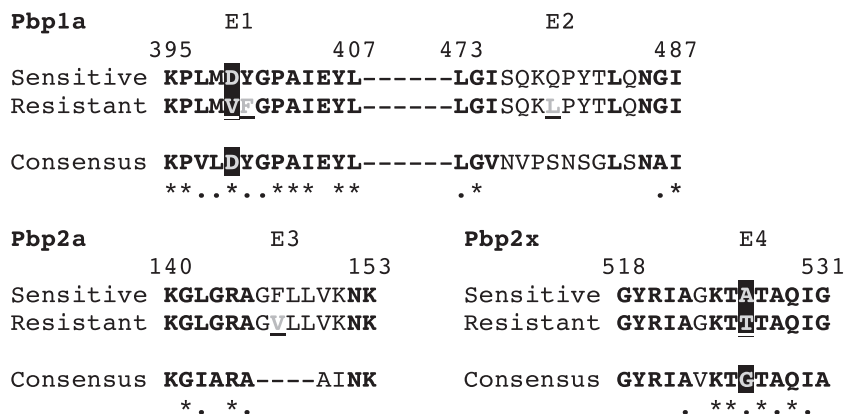
FIG. 1. Alignment of Pbp1a, Pbp2a, and Pbp2x from penicillin-sensitive and -resistant *L. reuteri* strains and the consensus sequences of Pbp from lactobacilli available in GenBank (10 species). Bold characters represent positions where the *L. reuteri* sequences are identical or have an amino acid residue with a function similar to that of the consensus sequence. Stars below the consensus sequence show positions where all *Lactobacillus* sequences are identical, and dots show positions where all have similar functions. The positions where resistant strains differs from sensitive strains (E1 to E4) are marked with gray and underlined characters, and the two substitutions (E1 and E4) that are located in conserved regions and lead to shifts in function are marked with a black background.

TABLE 4. Genes present on plasmids pLR581[a] and pLR585[b] and their putative functions

Plasmid and gene locus	Accession no.	Function
pLR581		
lr1991	EF421931	Putative replication protein
lr1992	EF421932	Putative replication protein
lr1993	DQ219980	Transcriptional regulator
lr1994	EF421933	Phage-related protein
lr1995	EF421934	DNA integrase/recombinase
lr1996	EF421935	Tetracycline resistance protein W [tet(W)]
lr1997	DQ074950	Truncated extracellular protein
lr1998	EU621694	Arsenite efflux pump ACR3, truncated
lr1999	DQ857855	Transcriptional regulator, ArsR
lr2000	EU621695	Arsenate reductase, truncated
lr2001	EU621696	Arsenite efflux pump ACR3
lr2002	DQ857856	Transcriptional regulator, ArsR
lr2003	EU621697	Plasmid-associated protein
lr2004	DQ857857	Replication initiator protein
pLR585		
lr2094	ACC61202	Recombinase
lr2095	ACC61203	Transcriptional regulator
lr2096	DQ857862	Polyketide antibiotic exporter
lr2097	DQ857863	Polyketide transporter, ATPase component
lr2098	ACC61204	DNA recombinase
lr2099	ACC61205	Plasmid-associated protein
lr2100	ACC61206	Plasmid-associated protein
lr2101	EF421942	Putative replication protein
lr2102	EF421943	Putative replication protein
lr2103	DQ219991	Putative transcriptional regulator
lr2104	ACC61207	Putative protease
lr2105	ACC61208	Lincomycin resistance protein [lnu(A)]
lr2106	ACC61209	Truncated mobilization protein
lr2107	ACC61210	Transposase, IS30 family

[a] Accession number EU583804.
[b] Accession number EU596446.

without sucrose, indicating efficient formation of protoplasts. Two hundred colonies from the sucrose plates were examined for growth or nongrowth on MRS and MRS-Tet agar. Seven nongrowing colonies were replated and grew on MRS-Tet, indicating that these colonies were false candidates. After 24 h of further incubation of the sucrose plates, a few more colonies appeared. Twelve such colonies were picked to MRS and MRS-Tet agar and grown overnight. One of these colonies grew well on the MRS plate but not at all on the MRS-Tet plate. This colony was replated, stored at −70°C, and designated L. reuteri ATCC 55730Tet[s].

L. reuteri ATCC 55730Tet[s] was then again subjected to protoplast formation in order to obtain a strain cured of pLR585. One hundred colonies from the sucrose plates were examined for growth or nongrowth on MRS and MRS-Lin agar. One colony was found to grow well on the MRS plate but not at all on the MRS plate with lincomycin. This colony was replated, stored at −70°C, and designated L. reuteri ATCC 55730Tet[s]Lin[s].

Confirmation of the loss of plasmids pLR581 and pLR585 was performed by PCR. In ATCC 55730Tet[s], lr2004 (gene encoding a replication protein on pLR581) and lr1996 [tet(W)] were both missing while the other ATCC 55730 plasmids and gene lr2105 [lnu(A)] could still be detected (Fig. 2A and B, lanes 2). Both lr2004 and lr2102 (gene encoding a replication protein on pLR585) were lacking in L. reuteri ATCC 55730Tet[s]Lin[s], whereas the genes representing the other two plasmids were still present (Fig. 2A, lane 3). Both tet(W) and lnu(A) was absent in ATCC 55730Tet[s]Lin[s] (Fig. 2B, lane 3). This clearly showed that L. reuteri ATCC 55730 had been cured of the tet(W)- and lnu(A)-containing plasmids. The double-cured ATCC 55730Tet[s]Lin[s] strain was later deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) as L. reuteri DSM 17938 (L. reuteri Protectis), and ATCC 55730Tet[s] was deposited as L. reuteri DSM 17686. The genes of the removed plasmids and the putative functions of the encoded proteins are listed in Table 4.

Comparative studies on L. reuteri ATCC 55730 and DSM 17938. The rep-PCR patterns obtained from strains ATCC 55730 and DSM 17938 were not possible to distinguish (Fig. 3), which shows not only that DSM 17938 is a true variant of ATCC 55730 and not a contaminant but also that removal of pLR581 and pLR585 did not affect the rep-PCR fingerprint.
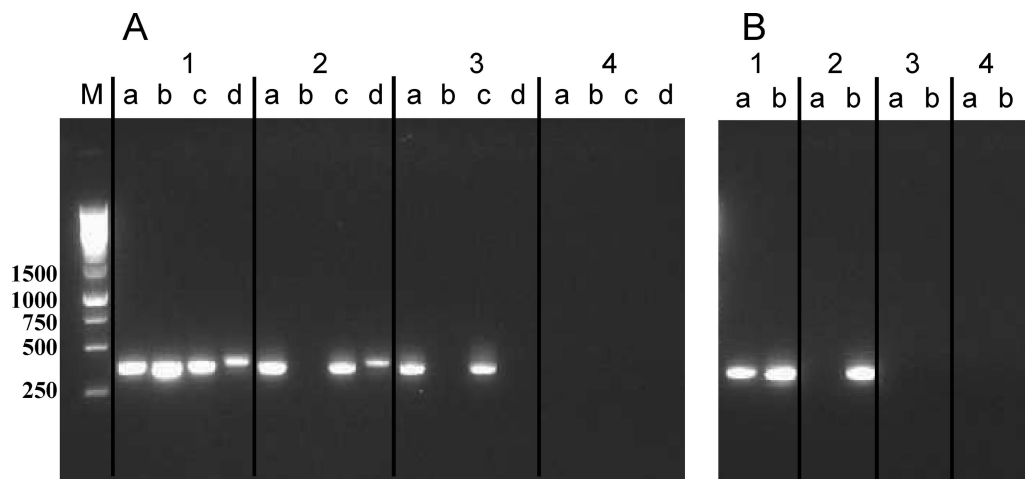

FIG. 2. (A) Detection of plasmids by PCR. Strains were analyzed for the presence of plasmids as follows: a, pLR580; b, pLR581; c, pLR584; d, pLR585. Lanes: 1, L. reuteri ATCC 55730; 2, L. reuteri ATCC 55730Tet[s]; 3, L. reuteri DSM 17938; 4, L. reuteri DSM 20016 (negative control). (B) Detection of tet(W) (a) and lnu(A) (b) by PCR. Lanes 1 to 4 are as in Fig. 1A. Lane M, molecular size markers (sizes are shown in base pairs on the left).
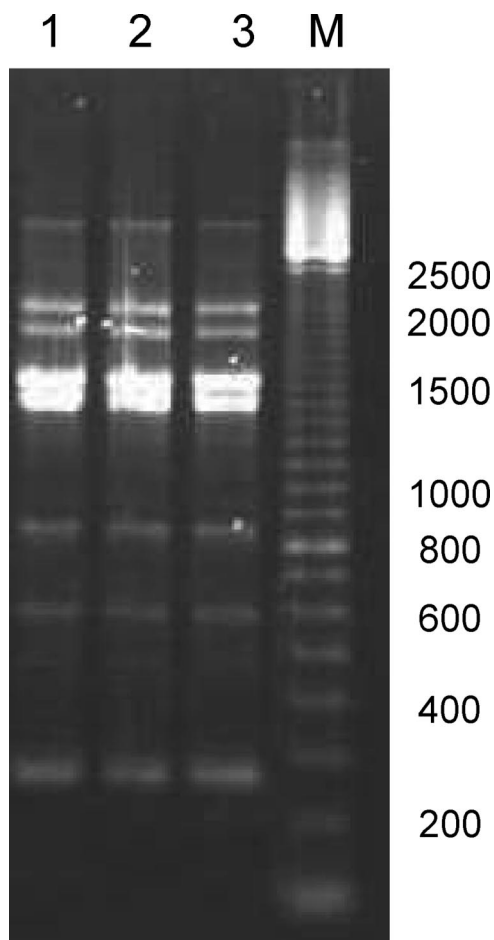
FIG. 3. (GTG)$_5$-PCR (rep-PCR)-generated genomic fingerprints of *L. reuteri* DSM 17938 (lane 1), *L. reuteri* ATCC 55730Tet^s (lane 2), and *L. reuteri* ATCC 55730 (lane 3). Lane M, molecular size markers (sizes are shown in base pairs on the right).

The MICs of tetracycline for the DSM 17938 and ATCC 55730Tet^s variants were found to be 12 to 16 μg ml^{-1} (Table 5), showing the natural intrinsic sensitivity of this species to tetracycline (18), compared to >256 μg ml^{-1} for ATCC 55730. This shows that the *tet*(W) gene is responsible for the unusually high tetracycline resistance of ATCC 55730. Strains ATCC 55730 and ATCC 55730Tet^s were both found to be resistant to lincomycin but not to clindamycin (a derivative of lincomycin). Removal of pLR585 resulted in a decrease in the lincomycin MIC for DSM 17938 from >16 to 0.25 μg ml^{-1}, while the sensitivity to clindamycin was unchanged. This confirms that the lincomycin resistance of ATCC 55730 was mediated by the

TABLE 5. MICs of tetracycline, lincomycin, and clindamycin for
L. reuteri strains

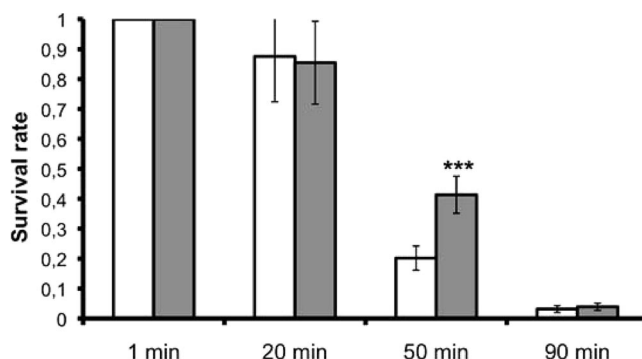| Strain	MIC (μg ml^{-1})		
	Tetracycline	Lincomycin	Clindamycin
ATCC 55730	>256	>16	<0.125
ATCC 55730Tet^s	12–16	>16	<0.125
DSM 17938	12–16	0.25	<0.125

FIG. 4. Acid tolerance of parent strain *L. reuteri* ATCC 55730 (light bars) and daughter strain *L. reuteri* DSM 17938 (dark bars). The columns show the proportion of bacteria surviving at pH 2.0 (mean of quadruplicates ± standard deviation). ***, $P < 0.001$ for 55730 versus 17938 (Student's *t* test).

pLR585 plasmid-borne *lnu*(A) gene. All other intrinsic resistances described for ATCC 55730 (18, 24) were unchanged in DSM 17938 (data not shown).

Fermentation patterns. The fermentation patterns showed that ATCC 55730 and DSM 17938 fermented L-arabinose, ribose, galactose, glucose, maltose, lactose, melibiose, saccharose, raffinose, and gluconate and were negative for the rest of the substrates. The production of reuterin was of the same magnitude for both strains, and there were no detectable differences in either colony or cell morphology or in mucus binding (data not shown).

Comparison of the growth of strains DSM 17938 and ATCC 55730 showed no difference in generation time, but DSM 17938 did grow to a significantly higher density than ATCC 55730 ($P < 0.01$). The final ODs of ATCC 55730 and DSM 17938 were 4.78 ± 0.13 and 6.00 ± 0.26, respectively, after 510 min of growth. Acidic challenge at pH 2.0 showed no difference after 20 and 90 min, but at the interim 50-min point, DSM 17938 survived significantly better than ATCC 55730, with survival rates of 41 and 20%, respectively (Fig. 4). Both ATCC 55730 and DSM 17938 tolerated bile well when they were in stationary phase. In different experiments, 30 to 90% of the cells grew on MRS agar with 6% bile. Survival and growth of both strains were lower in the exponential phase (0.1 to 1% growing cells). However, no difference in bile tolerance could be detected between the strains (data not shown).

Pathogen inhibition. Strains ATCC 55730 and DSM 17938 were both able inhibit the growth of *C. albicans*, *E. sakazakii*, *S. enterica* serovar Typhimurium, and *C. difficile*, and there was no difference between the strains in strength of inhibition in the presence or absence of glycerol (Table 6). In the absence of glycerol, neither strain showed any inhibitory effect on *C. albicans* growth.

Clinical trial. Compliance (participant self-reported) was 99% between days 1 and 14 and 97.5% between days 14 and 28. General health examinations revealed no alterations in weight, pulse, blood pressure, or body temperature in any group during the supplementation period, and blood safety and metabolic parameters were unchanged in any of the groups (data not shown). Blood taken directly after supplementation on day 28 was analyzed in 10 subjects, including 4 subjects from the

TABLE 6. Pathogen inhibition by *L. reuteri* strains with and without glycerol in the substrate

Indicator organism	Inhibition zone ± SD (mm)			
	ATCC 55730		DSM 17938	
	With glycerol	Without glycerol	With glycerol	Without glycerol
S. enterica serovar Typhimurium	43.7 ± 2.1	40.0 ± 2.0	42.7 ± 2.1	39.7 ± 2.1
E. sakazakii	42.0 ± 1.0	32.7 ± 1.5	41.0 ± 2.0	33.0 ± 1.0
C. difficile	41.0 ± 1.0	29.7 ± 0.6	40.7 ± 1.5	30.0 ± 1.0
C. albicans	41.0 ± 1.0	NI[a]	42.3 ± 1.5	NI

[a] NI, no inhibition.

high-dose 17938 group. All were negative for bacteremia. Thus, supplementation with *L. reuteri* DSM 17938 daily for 28 days at doses of up to 6.5×10^{10} CFU/day was well tolerated during the trial.

Three of the 16 individuals had detectable *L. reuteri* in their baseline fecal samples (Table 7). One of these isolates was 55730 like, while the others were unidentifiable *L. reuteri* strains. Standard ingested doses of *L. reuteri* ATCC 55730 and DSM 17938 led to similar levels of *L. reuteri* detection in the feces (Table 7). Increasing the dose of *L. reuteri* DSM 17938 100-fold led to 100-fold higher levels in the feces (Table 7). The levels detected were maximal at day 7 in all *L. reuteri*-supplemented groups and remained at this level during the period of supplementation. After a washout period of at least 2 weeks, there was no evidence of *L. reuteri* in the feces of any of the supplemented subjects (Table 7). Identification of fecal isolates by plasmid analysis obtained from samples taken during supplementation were found to correspond to the DSM 17938 plasmid pattern for all of the subjects ingesting this strain, with the exception of three samples where the strain was either ambiguous or resembled 55730. Isolates from the 55730 samples were all 55730 like, with the exception of two, which were ambiguous. Placebo-supplemented subjects showed no fecal colonization with *L. reuteri*, except for two observations of unknown *L. reuteri* in one individual.

DISCUSSION

β-Lactam resistance is conferred mainly by two mechanisms: drug inactivation by β-lactamases and target site (i.e., Pbp) alterations. For both types, transferable resistance determinants have been identified, e.g., *ampC* and *mecA*, respectively (21, 30). When the nature of the β-lactam resistance of *L.*

reuteri ATCC 55730 was investigated, point mutations in Pbp1a, Pbp2a, and Pbp2x were identified. Alterations in both Pbp1a and Pbp2x are often reported to cause resistance to β-lactams in streptococci, and high-level resistance is often caused by alterations in more than one of the proteins (14, 22). The Pbp are "housekeeping" proteins found in all bacteria. In *L. reuteri* ATCC 55730, the corresponding genes are all located on the chromosome and are not coupled to any mechanisms for transfer to other bacteria. In addition, there is no other gene present in the genome sequence with similarities to known penicillin resistance genes. We therefore suggest that the β-lactam resistance of *L. reuteri* ATCC 55730 is caused by a number of point mutations in the genes encoding Pbp1a, Pbp2a, and/or Pbp2x and that this resistance can be regarded as nontransferable.

The ribosome protection-type resistance gene *tet*(W) (5, 35) is one of the most widespread tetracycline resistance genes in environmental samples (4, 44). Lincosamide nucleotidyltransferases encoded by *lnu* (formerly *lin*) genes inactivate lincosamides by adenylation (9, 11), and the gene has been shown to occur on plasmids (29). Lincosamide antibiotics include lincomycin and clindamycin, a semisynthetic derivative of lincomycin (10). Coupled resistances to lincomycin and clindamycin seem to be associated with the *lnu*(F) gene but not with the *lnu*(A) gene, which only confers lincomycin resistance (2). Our finding that *L. reuteri* ATCC 55730 is not resistant to clindamycin is in accordance with others (18, 26) but not with the result of Kastner et al. (24). The reason for the difference is not known, but the method validation of Egervärn et al. (19) in accordance with the recent ACE-ART project in Europe indicates the reliability of this study. The resistance to lincomycin in *L. reuteri* ATCC 55730 is caused by *lnu*(A), located on pLR585, but both *L. reuteri* ATCC 55730 and *L. reuteri* DSM 17938 are sensitive to clindamycin.

Our data clearly indicate that *L. reuteri* ATCC 55730 harbors four plasmids and that two of them harbored the *tet*(W) and *lnu*(A) genes, respectively. The total number of plasmids is not in agreement with the report of Klein et al. (26), who concluded that the strain harbors six plasmids with sizes in the range of 5 to 24 MDa, which corresponds to approximately 7.7 to 37 kb. The difference is probably due to the methods used. Klein et al. detected the plasmids by agarose gel electrophoresis, which can give an overestimation of the number of plasmids since plasmids can be present in different forms and can thus give rise to several bands. Different curing techniques have been used on *Lactobacillus* species, including chemical curing with agents like novobiocin, sodium dodecyl sulfate,

TABLE 7. Fecal *L. reuteri* levels in subjects given either *L. reuteri* ATCC 55730 or DSM 17938 for 28 days

Group (no. of subjects)	Amt of *L. reuteri*[a]					
	Day 0	Day 7	Day 14	Day 28	Day 42	Day 56
17938 high (5)	<2[b]	7.14 (4)	6.23 (5)	7.26 (4)	<2	<2
17938 standard (4)	<2[b]	4.92 (3)	4.15 (4)	5.19 (2)	<2	<2[b]
55730 (3)	<2	4.91 (3)	4.26 (3)	4.40 (1)	<2	<2
Placebo (4)	<2[b]	<2	<2[b]	<2	<2	<2

[a] Log_{10} CFU g^{-1} (wet weight) feces. Values are the mean of the samples in which *L. reuteri* was detected (the number of samples is in parentheses). Variation between samples was large but similar for all data points.
[b] *L. reuteri* was detected in one of the subjects at 3.7 to 6.9 log_{10} CFU g^{-1}.

acridine dyes, and ethidium bromide or using high temperature or protoplast formation (12, 28, 32, 33, 36, 42, 43). We have tested all of these methods, and protoplast formation was the only one that succeeded in curing ATCC 55730 of the plasmids. Also, protoplast formation was considered to be an appropriate method since it does not involve genetically modifying the organism and does not induce mutations in the bacterial chromosome that might be a consequence of the use of other curing agents.

Besides the *tet*(W) gene, eliminated plasmid pLR581 harbors 13 other genes. Most of them fall into two categories: replication protein-encoding genes and arsenic resistance genes (several of the latter are truncated). The pLR585 plasmid also harbors 13 genes besides *lnu*(A), 2 of which encode polyketide antibiotic exporters (lr2096 and lr2097). Importantly, none of the genes on the plasmids have any connection or function of importance for the known probiotic characteristics of this strain.

L. reuteri DSM 17938 containing only two plasmids (pLR580 and pLR584) has the same rep-PCR profile, fermentation pattern, reuterin production, morphology, growth rate, adhesion to mucus, pathogen inhibition profile, and bile tolerance as *L. reuteri* ATCC 55730. The abilities of the daughter strain to grow to a higher density and to survive better under acidic conditions are probably related to a decreased burden with respect to DNA replication and thereby a higher competitive ability compared to the parent strain. *L. reuteri* ATCC 55730 has earlier been shown to tolerate passage through the human gastrointestinal tract, as shown by fecal shedding, and attaches and grows in the stomach, duodenum, and ileum of subjects ingesting the strain (20, 39). Laboratory analysis of both parent strain *L. reuteri* ATCC 55730 and daughter strain *L. reuteri* DSM 17938 showed at least similar acid and bile tolerance and mucus binding, predicting that DSM 17938 should not have a changed ability to colonize the entire human gastrointestinal tract compared to ATCC 55730. Fecal analysis after ingestion confirmed this and shows that *L. reuteri* DSM 17938 survives throughout the human gastrointestinal tract in the same way as *L. reuteri* ATCC 55730 and further that colonization is only temporary, as is common in probiotic strains. *L. reuteri* DSM 17938 could be unequivocally identified by the plasmid pattern, which showed a direct correlation between the ingested and shed strains. The blood safety parameters studied demonstrate that *L. reuteri* DSM 17938 has a safety profile similar to that of the *L. reuteri* ATCC 55730 strain. Furthermore, an analysis of the *L. reuteri* DSM 17938 genome annotation did not reveal any further gene or gene cluster known to be involved in virulence or antibiotic resistance (not shown).

In conclusion, we have demonstrated that *L. reuteri* ATCC 55730 could be cured of two independent plasmids carrying unwanted antibiotic resistance traits and that the resultant *L. reuteri* DSM 17938 strain did not lose any of its probiotic characteristics. This may be a valuable alternative for other true probiotic strains that are found to harbor plasmid-borne resistances.

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