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Genetic Basis of Tetracycline and Minocycline Resistance in Potentially Probiotic *Lactobacillus plantarum* Strain CCUG 43738

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The potentially probiotic strain *Lactobacillus plantarum* CCUG 43738, which displayed atypical phenotypic resistance to tetracycline (MIC, 512 μg/ml) and minocycline (MIC, 256 μg/ml), was found to contain a *tet*(S) gene located on a plasmid of approximately 14 kb. Plasmid curing with novobiocin eliminated this plasmid and restored the tetracycline-susceptible phenotype of the host strain.

Lactic acid bacteria (LAB), such as lactobacilli and bifidobacteria, are increasingly being used as probiotics for humans. Within the large spectrum of probiotic claims and applications, there is a growing trend of focusing on specific clinical disorders such as irritable bowel syndrome. Recently, Wynne and coworkers (14) reported the isolation of a fecal tetracycline-resistant *Lactobacillus plantarum* strain displaying strong inhibitory in vitro effects against *Candida albicans*. The authors suggested that this strain may be useful as a probiotic in the management of yeast (*Candida*)-related clinical conditions, such as irritable bowel syndrome, provided that it fulfills the necessary selection criteria. In addition to functional and technological properties, it has been recommended that the selection process of a probiotic microorganism should also address a number of safety aspects (12). Because the absence of acquired antibiotic resistances is one of the first safety criteria to be checked in probiotic candidates, the current study aimed to unravel the genetic basis of tetracycline resistance in the potentially probiotic *L. plantarum* strain.

The taxonomic identity of the *L. plantarum* strain, which was deposited in the Culture Collection of the University of Göteborg, Sweden (http://www.ccug.se/) as CCUG 43738, was reconfirmed by protein profiling (11). Susceptibility to seven antimicrobial agents was tested using the broth microdilution method and Iso-sensitest (Oxoid Ltd., Basingstoke, United Kingdom) medium at 37°C under microaerobic conditions. PCR detection of *tet* genes and *int*-Tn*916* was performed as described previously (8). Oligonucleotide primers for sequencing of *tet*(S) were developed by using Kodon software (Applied Maths, Sint-Martens Latem, Belgium). Sequencing was performed by using the BigDye Terminator (version 3.1) ready reaction cycle sequencing kit on the ABI Prism 3100 genetic analyzer (Applied Biosystems). Plasmid profiling was performed as previously described (1), except for the fact that Tris-phosphate electrophoresis buffer was used and the electrophoresis time was 5 h. A supercoiled DNA ladder (Invitrogen) with a size range of 2 to 16 kb was used to size the plasmids. The DNA probe used in Southern blotting was labeled with horseradish peroxidase using the ECL direct nucleic acid labeling kit (RPN3000; Amersham Biosciences) according to the manufacturer's instructions. Plasmid curing was carried out by incubating the *L. plantarum* strain for 72 h with 0.5 to 1 μg/ml novobiocin. Filter mating was performed as previously reported (8) by using the following selective conditions: 10 μg/ml tetracycline, 50 μg/ml rifampin, and 100 μg/ml fusidic acid. The *L. plantarum* LMG 21687 strain, which contains a transferable *tet*(M)-carrying plasmid (7), was used as positive control strain in filter mating.

The following MICs were obtained for strain CCUG 43738 by using broth microdilution: 512 μg/ml (tetracycline), 256 μg/ml (minocycline), 8 μg/ml (streptomycin), <0.5 μg/ml (gentamicin), 0.25 μg/ml (erythromycin), 0.5 μg/ml (ampicillin) and <0.12 μg/ml (clindamycin). Although no MIC breakpoints have been defined for lactobacilli, a comparison with previous data (4) indicates that strain CCUG 43738 should probably be considered as susceptible to erythromycin, ampicillin, and clindamycin and displays atypical resistance to tetracycline. The finding of high-level resistance to both tetracycline and minocycline suggested that an acquired ribosomal protection mechanism was involved (3). Of the most commonly detected ribosomal protection-type *tet* genes in gram-positive bacteria, i.e., *tet*(M), *tet*(O), *tet*(Q), *tet*(S), and *tet*(W), PCR detection revealed that strain CCUG 43738 harbored the *tet*(S) gene but none of the other four *tet* genes tested. To our knowledge, tetracycline resistance in *L. plantarum* has so far been associated with the presence of only *tet*(M) (5, 6), and the present study is the first to document the prevalence of *tet*(S) in this species or in a fecal *Lactobacillus* strain.

The nearly complete sequence (nucleotide positions 68 to 1872, encompassing 93.8% of the 1,923-bp coding sequence) of *tet*(S) was determined for strain CCUG 43738 and for four other *tet*(S)-positive LAB from human feces and compared with two *tet*(S) sequences available from EMBL/GenBank. Whereas the *tet*(S) genes previously documented in *Lactococcus lactis* subsp. *lactis* strain 214 (accession no. Y10522) and *Listeria monocytogenes* strain BM4210 (accession no. L09756) or recently found in new isolates of *Enterococcus faecalis* strains D17 (accession no. AM039490) and V35 (accesssion no. AM039489), *Enterococcus durans* strain S42 (accession no.

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AM039488), and *Lactococcus garvieae* strain S22 (accession no. AM039487) were nearly identical, showing 99.9% sequence homology, the *tet*(S) gene of strain CCUG 43738 (accession no. AM039486) displayed a slightly lower sequence homology of 99.7% with all of the above-mentioned sequences. Relative to each of these six other *tet*(S) sequences, the *tet*(S) gene of strain CCUG 43738 displayed four codon substitutions (i.e., Glu324→Lys324, Pro442→His442, Gln476→Lys476, and Phe533→Leu533), suggesting that its evolutionary history may be slightly different from that of each of these other *tet*(S) genes.

Plasmid profiling revealed that strain CCUG 43738 contained at least eight plasmids in the range of 2 to >16 kb. Four plasmids had sizes larger than 16 kb, whereas the estimated sizes of the other four plasmids were 2, 5, 8.5, and 14 kb. Southern hybridization with a 700-bp amplification product of *tet*(S) as a probe revealed that this gene was located on one of these plasmids with an estimated size of 14 kb. Also, in previous studies, *tet*(S) has been detected on plasmids (2, 10) but it can also be integrated in a Tn*916*-like conjugative transposon (9). However, a negative PCR result for the *int* gene of Tn*916* suggests that no such element is present in strain CCUG 43738. By plasmid curing with novobiocin, multiple tetracycline-susceptible derivatives of strain CCUG 43738 were obtained that showed significantly reduced MICs for tetracycline and minocycline of 16 μg/ml and 4 μg/ml, respectively. The observed reduction in MIC for tetracycline from 512 to 16 μg/ml agrees with the MIC range of 2 to 32 μg/ml that was previously reported for tetracycline-susceptible *L. plantarum* strains (4). Further molecular characterization of these derivatives revealed that the 14-kb plasmid was eliminated, which resulted in the loss of the *tet*(S) gene, as demonstrated by PCR detection and Southern blotting. The conjugal transfer of the *tet*(S) plasmid from strain CCUG 43738 to *E. faecalis* recipient JH2-2 could not be demonstrated by filter mating. However, this finding does not exclude the possibility that this plasmid is transferable to other (phylogenetically more closely related) recipients.

The detection of plasmid-located *tet*(S) in *L. plantarum* CCUG 43738 confirms previous studies demonstrating that lactobacilli from food, feed, or fecal origin can harbor acquired antibiotic resistances (5, 6, 13), which is not considered a desirable trait for (potentially) probiotic strains from a safety point of view. As an alternative to the immediate removal of such strains from the probiotic selection process, the key message from this study is that a better understanding of the genetic basis of acquired resistances in health-promoting or starter LAB can stimulate the design of curing strategies to eliminate atypical resistances.

Nucleotide sequence accession numbers. The nucleotide sequences of *tet*(S) genes determined in this study have been submitted to the EMBL/GenBank sequence databases and assigned accession no. AM039486 to AM039490.

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