An L-fucose operon of probiotic *Lactobacillus rhamnosus* GG involved in adaptation to gastrointestinal conditions

Jimmy E. Becerra, María J. Yebra and Vicente Monedero*

Laboratory of Lactic Acid Bacteria and Probiotics, Biotechnology Department, Institute of Agrochemistry and Food Technology (IATA-CSIC), Paterna, Valencia, Spain

*Corresponding author.

E-mail: btcmon@iata.csic.es

Tlf: +34 963900022

Fax: +34 963636301

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ABSTRACT

L-fucose is a sugar present in human secretions as part of human milk oligosaccharides, mucins and other glycoconjugates in the intestinal epithelium. The genome of the probiotic *Lactobacillus rhamnosus* GG (LGG) carries a gene cluster encoding a putative L-fucose permease (*fucP*), L-fucose catabolic pathway (*fucI, fucK, fucU* and *fucA*), and a transcriptional regulator (*fucR*). The metabolism of L-fucose in LGG results in 1,2-propanediol production and their *fucI* and *fucP* mutants displayed a severe and mild growth defect on L-fucose, respectively. Transcriptional analysis revealed that the *fuc* genes are induced by L-fucose and subject to a strong carbon catabolite repression effect. This induction was triggered by FucR, which acted as a transcriptional activator necessary for growth on L-fucose. LGG utilized fucosyl-α1,3-N-acetylglucosamine and contrarily to other lactobacilli, the presence of *fuc* genes allowed this strain to use the L-fucose moiety. In *fucI* and *fucR* mutants, but not in *fucP* mutant, L-fucose was not metabolized and it was excreted to the medium during growth on fucosyl-α1,3-N-acetylglucosamine. The *fuc* genes were induced by this fucosyl-disaccharide in the wild-type and *fucP* mutant but not in a *fucI* mutant, evidencing that FucP does not participate in the regulation of *fuc* genes and that L-fucose metabolism is needed for FucR activation. The L-fucose operon characterized here constitutes a new example of the many factors found in LGG that allow this strain to adapt to the gastrointestinal conditions.

Keywords: *Lactobacillus rhamnosus* GG, intestinal microbiota, L-fucose, α-L-fucosidases, glycoconjugates
L-fucose (6-deoxy-L-galactose) is one of the few hexoses with L configuration found in nature. It forms part of many glycans present at the surface of eukaryotic cells such as H and Lewis antigens, which are present not only in blood cells but also in epithelial cells at different mucosal sites (1). It is also present at the highly glycosylated mucin proteins of the intestinal mucosa and in a high proportion of the oligosaccharides present in human milk (2, 3). These facts make L-fucose an important sugar in the microbial ecology of the gastrointestinal tract. The importance of the fucosylation of mucosal glycoconjugates on the intestinal ecology is reflected by the fact that the intestinal microbiota composition is dependent on the secretor status of the individuals, which is defined by mutations in the FUT2 gene coding for an α(1,2) fucosyltransferase that participates in the fucosylation of mucosal glycans (4, 5). Owing to its elevated concentration found in the intestinal niche, L-fucose can be used as a carbon source and its utilization has been identified as a key factor for intestinal colonization in some bacteria. Thus, the pathogen Campylobacter jejuni, primarily thought to be an asaccharolytic microorganism, was able to use L-fucose and this provides it with a competitive advantage, as determined in a neonatal piglet infection model (6). Also, a ΔfucAO mutant of the probiotic E. coli Nissle 1917 strain, unable to use L-fucose, showed two orders of magnitude lower colonization level in mouse intestine compared to the wild-type (7).

L-fucose metabolism has been extensively characterized in Escherichia coli (8-10). In this bacterium L-fucose is transported by a permease and catabolized to fuculose-1-phosphate that is finally split into dihydroxyacetone-phosphate and L-lactaldehyde by a specific aldolase. L-lactaldehyde can be further metabolized to 1,2-propanediol or lactate and the dihydroxyacetone-phosphate enters glycolysis (10). A similar pathway has been
described in *Bacteroides* (11). However, *C. jejuni* or intestinal commensals like *Bifidobacterium* rely on a different route for its catabolism, which involves L-fucose dehydrogenase and L-fuconolactonase, although the corresponding genes and enzymes have not been fully characterized (6).

L-fucose does not only serve as a carbon and energy source, but in some bacteria it acts as a signaling molecule. In enterohaemorrhagic *E. coli* the two-component system FusKR senses extracellular L-fucose and activates virulence and metabolic genes (12). *Streptococcus pneumoniae* strains, although having all the genes which encode a complete L-fucose pathway are not able to use it. The *S. pneumoniae fuc* genes are clustered with genes encoding extracellular glycosidases that release fucosyl-oligosaccharides from LewisY and type A and B histo-blood group antigens from the host cell surface. These fucosyl-oligosaccharides are taken up by specific transporters encoded in the same operon. The *S. pneumoniae fuc* genes are needed for virulence and are induced by L-fucose but this sugar is not further metabolized (13).

*Lactobacillus rhamnosus* GG (LGG) is a bacterium marketed as probiotic (14). It was derived from the intestinal habitat of a healthy human and its health benefits as probiotic have been widely documented (14, 15). Genetic and biochemical studies have revealed that LGG possesses many mechanisms of survival and persistence at the gastrointestinal tract (16), which include bile salt resistance (17), proteic factors for mucosal attachment (e.g. mucus binding pili (18)), glycosidases, sugar transporters and catabolic enzymes needed to exploit the carbohydrate resources characteristic of the intestinal habitat (19). LGG is able to use L-fucose as a carbon source and putative L-fucose utilization genes are annotated in its genome, but their functionality has never been proved, and no data on L-fucose utilization by intestinal lactobacilli are available. In this work the involvement of LGG *fuc* genes in L-fucose utilization has been established,
revealing a new trait that may be important in the persistence and colonization of the intestinal habitat by LGG.
MATERIAL AND METHODS

Strains and growth conditions

*Lactobacillus* strains (Table 1) were grown in MRS medium (Difco) or in basal MRS medium (10 g/liter Bacto peptone (Difco), 4 g/liter yeast extract (Pronadisa), 5 g/liter sodium acetate, 2 g/liter triammonium citrate, 0.2 g/liter magnesium sulfate 7-hydrate, 0.05 g/liter manganese sulfate monohydrate, and 1 ml/liter Tween 80) supplemented with 0.5% L-fucose (30 mM) or 4 mM fucosyl-α1,3-N-acetylglucosamine (Fuc-α1,3-GlcNAc, Carbosynth Ltd, Compton, Berkshire, UK) at 37ºC under static conditions. Bacterial growth was determined in microtitre plates in a Polarstar Omega plate reader at 37ºC. Each well (100 µl medium) was inoculated with bacteria grown in basal MRS without sugars at an initial OD 550nm of 0.1. The maximum growth rate (μ) was calculated by following the OD 550nm versus time. Three independent biological replicates for each growth curve were obtained. Anaerobic and aerobic growth of LGG was carried out in 10 ml of basal MRS supplemented with 20 mM L-fucose or 20 mM glucose in anaerobic jars (AnaeroGen, Oxoid) or in 50 ml Erlenmeyer flasks shaken at 200 rpm, respectively. The medium was inoculated at an initial OD 550nm of 0.1 and incubated at 37ºC for 24 h. *E. coli* DH10B was used as cloning host and it was grown in LB at 37ºC under shaking at 200 rpm. Antibiotics used for plasmid selection were ampicillin at 100 µg/ml and chloramphenicol at 20 µg/ml for *E. coli* and erythromycin at 5 µg/ml for LGG.

Construction of *L. rhamnosus* GG mutants in *fuc* genes

LGG mutants in *fucP* (LGG_02683) and *fucI* (LGG_02685) were constructed by replacing the wild-type genes with mutated variants. The LGG chromosomal DNA was isolated from 10 ml cultures with the DNA Isolation Kit for Cells and Tissues (Roche). The *fucP*
gene was amplified by PCR with Pfx DNA polymerase (Invitrogen) and the oligonucleotide pair FucP1 (5'-GCCTTGCGATGGTCTATAG)/FucP2 (5'-GCTTCTTCTCAGTTGATCAAC) using the isolated chromosomal DNA as template. The resulting 2187-bp fragment was digested with AclI and the 634- and 590-bp fragments corresponding to 5' and 3' portions of fucP, respectively, were gel-isolated. These fragments were ligated together with EcoRV-digested pRV300 (20), creating an integrative plasmid carrying a fucP gene with an internal 963-bp in-frame deletion (pRΔFucP). The fucI gene was amplified by PCR with oligonucleotides FucI1 (5'-ATTGGCGCGTTGAATGAAAG) and FucI2 (5'-ATAAGATCCGGCTGGTTTCC) and the obtained fragment was digested with PstI. The generated 997-bp fragment was gel isolated and ligated to pRV300 digested with EcoRV/PstI. The obtained plasmid was digested with EcoRI, treated with Klenow and ligated, creating thus a frameshift in fucI as was verified by sequencing (pRFucI plasmid). The plasmids containing the mutated fucP and fucI genes were transformed by electroporation in LGG with a GenePulser apparatus (Biorad) as previously described (21). Integrants obtained by single cross-over recombination were selected on MRS agar plates containing erythromycin. Clones were grown for about 200 generations in antibiotic-free MRS medium and plated on MRS. Colonies that had undergone a second recombination event leading to an erythromycin-sensitive phenotype were selected by replica-plating and tested by PCR with appropriated oligonucleotides for the replacement of the wild type genes by the mutated copy. After confirmation of the mutations by sequencing, two clones were selected and named BL394 (ΔfucP) and BL395 (fucI).

A 437-bp internal fragment of the fucR gene (LGG_02680) was amplified with oligonucleotides FucR1 (5'-GGCGTGGCTTTGGATATG) and FucR2 (5'-CATCATCGCTCGCTTGAC) and cloned into pRV300 digested with EcoRV. The
resulting plasmid (pRFucR) was used to transform *L. rhamnosus* GG selecting for erythromycin resistance. One strain with disrupted *fucR* was selected and named BL396.

RT-qPCR analysis of *fuc* genes expression

*L. rhamnosus* strains were grown in MRS basal medium containing 0.5% glucose, 0.5% L-fucose or 0.5% L-fucose plus 0.2% glucose to an OD$_{590}$ of 0.9-1. Bacterial cells from 9 ml cultures were recovered by centrifugation and washed with 9 ml of EDTA 50 mM pH 8. The cell pellets were resuspended in 1 ml or TRIzol reagent (Gibco) and one gram of glass beads (0.1 mm diameter) were added. The bacteria were broken with a Mini BeadBeater apparatus (Biospec Products, Bartlesville, OK) and total RNA was isolated following the recommendations of the manufacturer of TRIzol. For experiments with Fuc-$\alpha_1,3$-GlcNAc, RNA was isolated from 1 ml cultures containing 4 mM sugar. One hundred nanograms of RNA were digested with DNase I (RNase free, Fermentas), and cDNA was obtained from 50 ng of DNase-treated RNA using the Maxima First Strand cDNA synthesis kit (Fermentas). qPCR was performed in a LightCycler 480 II system (Roche) with the LightCycler FastStart DNA Master SYBR Green I mix (Roche). Primers were designed by using the Primer-BLAST service at the NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) in order to generate amplicons ranging from 70 to 100 bp in size. qPCR was performed for each cDNA sample with the primers pairs 5’-TGGCTAAGCATGGGTTCTTG/5’-TCGCCATTGATCGTCTCT (fucI), 5’-CCGGAGGCCAGTCAAAGTT/5’-CTCAACAGGCCAGCTACA (fucK), 5’-TTTGTCGGCTGACACGATGA/5’-GCCGGCGCTAATTCAGAAAG (fucP), 5’-GAGCAGATGGCCTGACAGTT/5’-ACTGGCGGTTCACCATTAGG (fucU), and 5’-GAAGTTGCTGGTGCAAAGGG/5’-TCATCCGGGTATTCGCTGTG (fucA), and 5’-TCAGTTGTGCAGTGCAGT/5’-TTCAACGCCTGCATCTGCTA (fucR). The reaction
mixture (10 μl) contained 5 μl of 2X qPCR master mix, 0.5 μl of each primer (10 μM), and 1 μl of a 10-fold dilution of the cDNA synthesis reaction. Reaction mixtures without a template were run as controls. The cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of three steps consisting of denaturation at 95°C for 10 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 20 s. For each set of primers, the cycle threshold (crossing point [CP]) values were determined by the automated method implemented in the LightCycler software (version 4.0; Roche). The relative expressions were calculated using the software tool REST (relative expression software tool) (22) and three LGG reference genes (pyrG (LGG_02546), recG (LGG_01660) and leuS (LGG_00848)) were used simultaneously for the analysis. Linearity and amplification efficiency were determined for each primer pair and every RT-qPCR reaction was performed at least in triplicate with two biologically independent samples.

Sugar and metabolite analysis

The concentration of L-fucose and Fuc-α1,3-GlcNAc in the supernatants was determined by high-pH anion-exchange chromatography with pulsed amperometric detection in an ICS3000 chromatographic system (Dionex) using a CarboPac PA100 column (Dionex). A combined gradient of 100 to 300 mM NaOH and 0 to 150 mM acetic acid was used for 20 min at a flow rate of 1 ml/min. 1,2-propanediol and lactic acid were determined by HPLC with a Jasco PU-2080Plus system coupled to refractive index (Jasco RI-2031Plus) or UV (210 nm) detectors with Rezex RCM-Monosaccharide and Rezex ROA-Organic Acid columns, respectively. Flow rates were 0.6 ml/min and 0.5 ml/min in water or 5 mM H2SO4, respectively.
Sequence analysis

The sequences of the LGG fuc genes were retrieved from the GenBank database (acc. No FM179322.1) and homology searches were performed with BLAST at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Genomic context analysis was performed on genomes deposited at the Microbial Genome Database for Comparative Analysis (http://mbgd.genome.ad.jp/) (23). Transcriptional terminators in the fuc gene cluster were searched with ARNold (http://rna.igmors.u-psud.fr/toolbox/arnold/).

Statistical analysis

Statistical analysis was performed using GraphPad Software (San Diego, CA). Student's t-test was used to detect statistically significant differences between growth rates from the wild type strain and the mutant strains. Statistical significance was accepted at $P < 0.05$. 
RESULTS

The L. rhamnosus GG fuc operon

An L-fucose operon is annotated in the genome of LGG (LGG_02680 to LGG_02685) which codes for the enzymes that would comprise a complete pathway for L-fucose catabolism (Figure 1). The genes fucI, fucK, fucP and fucU would code for an L-fucose isomerase, L-fuculose kinase, an L-fucose/H⁺ symporter of the major facilitator superfamily (MFS) and L-fucose mutarotase, an enzyme which accelerates the conversion of the β anomer of L-fucose into the α anomer, the substrate for FucI (24).

The divergently transcribed fucR gene codes for a transcriptional regulator of the DeoR family, whereas fucA, codes for an L-fuculose-1-phosphate aldolase which splits L-fuculose-1-phosphate into dihydroxyacetone-phosphate and L-lactaldehyde, and it is transcribed in the opposite direction to fucIKPU. In E. coli the L-lactaldehyde formed during L-fucose catabolism is detoxified by the action of an L-1,2-propanediol oxidoreductase encoded by a gene in the fuc cluster, fucO, with the concomitant production of 1,2-propanediol (10). No fucO homologue is found in the LGG fuc cluster but two genes which encode proteins with 50% (LGG_00757) and 42% identity (LGG_02124) to E. coli FucO are present at another location in the LGG chromosome.

Alternatively, in E. coli L-lactaldehyde can be transformed into lactic acid by the action of lactaldehyde dehydrogenase, encoded by the aldA gene (9). In LGG the gene LGG_02286 codes for a protein with 32% identity to E. coli AldA, which possesses the typical motifs of NAD(+) -dependent aldehyde dehydrogenases.

fuc clusters encoding similar catabolic pathways are found in the genomes of some intestinal commensals and pathogens although the genetic structure varies and fucO and fucU genes are not always present (Figure 2). A second type of transcriptional regulator, with N-terminal DNA binding domain of the GntR superfamily and a C-terminal ligand-
binding domain of the sugar-binding domain of ABC transporters, replaces the DeoR-
family FucR in *Bacteroides* (11). Furthermore, the *fuc* operons from *S. pneumoniae*
strains are more complex and they lack the gene for an L-fucose/H⁺ symporter
permease, which is replaced by genes encoding phosphoenolpyruvate: sugar
phosphotransferase systems (PTS) of the mannose-class or sugar ABC-type
transporters, and contain genes for intracellular and extracellular glycosyl hydrolases
(Figure 2) (13).

Genome inspection in the rest of *Lactobacillus* species revealed that *fuc* clusters are
exclusively found in some *L. rhamnosus* isolates (e.g. GG, HN001, LRHMDP2 and
LRHMDP3 strains), *Lactobacillus casei* and *Lactobacillus zeae* (only one sequenced
representative strain is available for these species: strains ATCC393 and ATCC15820,
respectively). According to this, ATCC15820 strain was able to ferment L-fucose (data
not shown). By the contrary, ATCC393 strain did not form acid from L-fucose (data not
shown). This inability is probably due to the presence of a frame-shift in the *fucK* gene of
this strain (resulting in two different annotated genes: LBCZ_2453 and LBCZ_2454) that
gives truncated L-fuculose kinases lacking the N-terminal 328 and C-terminal 181 amino
acids, respectively. Among the rest of lactobacilli, homologues to some *fuc* genes were
only found in the draft genomes of newly described species: *Lactobacillus
shenzhenensis* LY-73T, *Lactobacillus composti* JCM14202 and *Lactobacillus herbinensis*
DSM16991 (data not shown). However, they were not grouped in an identifiable *fuc*
cluster. It seems therefore that within *Lactobacillus* the presence of a complete set of *fuc*
genes organized in an operon structure is exclusive for *L. rhamnosus* and *L. casei /
zeae*. Despite of the elevated number of sequenced strains of *Lactobacillus paracasei*
(25), no *fuc* genes were found in this species, which forms part of the phylogenetically-
related *L. casei / paracasei / rhamnosus* group.
Another remarkable feature of the LGG \textit{fuc} region was the presence of an adjacent set of genes (LGG_02687 to LGG_02692) encoding an L-rhamnose-1-phosphate aldolase, L-rhamnose isomerase, L-rhamnose mutarotase, L-rhamnulokinase, a MFS permease and a transcriptional regulator of the AraC family, which would constitute a complete pathway for L-rhamnose (6-deoxy-L-mannose) utilization, analogous to the L-fucose catabolic pathway. Therefore, it appears that this chromosomal region in LGG consist of genes for the catabolism of L-deoxy-sugars.

\textbf{The mutation in \textit{fuc} catabolic genes impairs growth on L-fucose}

In order to prove the involvement of \textit{fuc} genes in L-fucose catabolism we constructed mutants in \textit{fucI} and \textit{fucP} (Figure 1A). The \textit{fucI} strain failed to acidify the growth medium when it was supplemented with L-fucose, whereas in the \textit{fucP} mutant acidification was slower compared to the wild type (data not shown). Under our experimental conditions, LGG showed a slow growth in non-supplemented basal MRS medium, probably due to the consumption of residual carbon sources present in this complex medium, as has been described earlier (26). When the medium was supplemented with L-fucose the wild-type strain showed a growth rate (0.114 ± 0.022 h⁻¹), compatible with L-fucose utilization, whereas the \textit{fucI} mutant strain displayed a growth rate significantly lower than that of the wild type (0.077 ± 0.007 h⁻¹, \( P = 0.048 \)) and lower ODs were reached (Figure 3A). In the \textit{fucP} mutant L-fucose was only able to sustain a reduced growth rate (0.078 ± 0.006 h⁻¹, \( P = 0.048 \)), but the attained ODs were higher compared to the \textit{fucI} strain (Figure 3B), suggesting that, although FucP is the main L-fucose transporter in LGG, in the absence of this permease L-fucose is probably entering the cells by alternative and less efficient transporter(s). In order to determine if, similar to \textit{E. coli} (10), the fate of L-fucose in LGG depends on the oxygen availability, the wild-type supernatants were analyzed after
growth under anaerobic or aerobic conditions. The analysis evidenced the production of 1,2-propanediol when cells were grown with L-fucose under anaerobiosis (Table 2). This compound was not detected in supernatants of glucose-grown cells. This confirmed that in LGG the L-lactaldehyde formed by the action of the L-fuculose-1-phosphate aldolase on L-fuculose-1-phosphate can be metabolized by an L-1,2-propanediol oxidoreductase as occurs in E. coli. Lactate production from L-fucose under anaerobic conditions was lower compared to cells grown with glucose. Aerobic conditions did not have a strong impact on lactic acid production or growth with glucose. However, these conditions did not favor L-fucose utilization, which was very inefficient and incomplete after 24 h (Table 2).

The fucR gene codes for a transcriptional activator of the fuc cluster

To ascertain the role of the product of fucR in the regulation of the fuc operon a disrupted mutant was constructed (Figure 1A). Similar to the futl mutant, the fucR mutant strain displayed a growth rate (0.067 ± 0.013 h⁻¹, P = 0.032) significantly lower than that of the wild type. Thus, fucR mutant failed to grown with L-fucose as a carbon source (Figure 3C), supporting the role of FucR as a transcriptional activator of fuc genes. Gene expression analysis of the fuc operon revealed that all fuc genes were induced by the presence of L-fucose by a factor ranging from 12 (fucR) to 9400 (fucU) compared to growth on glucose (Figure 4). According to the gene structure of the LGG fuc cluster (Figure 1A), at least three different promoters are necessary for the transcription of all genes. Our results show that the three promoters are responsive to L-fucose and suggested that FucR autoregulates its own transcription, although fucR displayed the lower level of induction compared to the rest of fuc genes.
The presence of glucose in addition to L-fucose caused a strong carbon catabolite repression of all fuc genes, whose expression was restored to levels close to those found during growth on glucose, with a factor of repression (fold-change in L-fucose/fold-change in L-fucose plus glucose) ranging from 30- to 4400-fold. In agreement with this observation catabolite responsive element (cre) sites which fitted the consensus sequence defined for Lactococcus lactis (WGWAARGYTWWMA, (27) were found upstream of fucI (−86TGAAAGCGCTTATT, two mismatches) or fucA and fucR (−57TGCAAGCGCTTACG, one mismatch; the numbering is relative to fucR). These sites are the target for binding of the global transcriptional regulator CcpA, which controls carbon catabolite repression in Low-G+C Gram-positive bacteria (28).

**Growth of L. rhamnosus GG with a fucose-containing disaccharide**

A previous work has shown that Lactobacillus casei BL23 is able to utilize the fucosyl-disaccharide fucosyl-α1,3-N-acetylglucosamine (Fuc-α1,3-GlcNAc), which is transported by a specific permease of the PTS class and split into L-fucose and N-acetylglucosamine by the α-L-fucosidase AlfB (29). However, this strain is not able to use L-fucose and only the N-acetylglucosamine moiety of Fuc-α1,3-GlcNAc is catabolized, while L-fucose is quantitatively expelled to the growth medium. LGG was also able to grow with 4 mM Fuc-α1,3-GlcNAc (Figure 5) and, in agreement with the presence of an L-fucose catabolic pathway, no L-fucose was found in the supernatants. By the contrary, compared to the wild type the LGG fucI and fucR mutants reached a lower final OD on Fuc-α1,3-GlcNAc (Figure 6). In these experiments 110±6.1 % (fucI mutant) and 70.8±1.4 % (fucR mutant) of the theoretical L-fucose from Fuc-α1,3-GlcNAc (4 mM) was detected in the bacterial supernatants at the stationary phase. This was consistent with the fact that only the N-acetylglucosamine moiety of the disaccharide was efficiently metabolized in these mutants. The fucP mutant, deficient in the L-fucose permease, showed a growth pattern
on Fuc-α1,3-GlcNAc similar to the wild type (Figure 5) and only 3.1±0.3 % of the theoretical L-fucose generated from Fuc-α1,3-GlcNAc was detected in supernatants. These data indicated that the L-fucose intracellularly generated, possibly by the action of an AlkB homologue (coded by LGG_02652), was metabolized, and that FucP was not fully necessary for this process. In agreement with this, growth on Fuc-α1,3-GlcNAc induced expression of all fuc genes, although the use of lower concentrations of disaccharide (4 mM) led to lower induction levels (Figure 6) compared to growth with 0.5% (30 mM) L-fucose (Figure 4). Also, under these conditions the fucR gene showed a downregulation compared to cells grown with glucose. In the ΔfucP strain expression of the fuc cluster after growth with fuc-α1,3-GlcNAc was comparable to that found in the wild type, further supporting the fact that fucP is dispensable for Fuc-α1,3-GlcNAc metabolism. In the fucI and fucR mutants no induction of fuc genes was observed after growth on Fuc-α1,3-GlcNAc (Figure 6), which was in agreement with the observed excretion of L-fucose in these strains when cells were grown on the fucosyl-disaccharide.
DISCUSSION

L. rhamnosus GG (LGG) is widely used as probiotic and this strain has the ability to survive and transiently colonize the gastrointestinal tract in animal models and humans (16, 19). In this context, the presence in LGG of genes involved in the utilization of host-derived glycans constitutes a competitive advantage for its persistence in the gut. LGG codes for about 40 putative glycosidases, many carbohydrate transporters and catabolic enzymes that would allow it to take advantage of the carbohydrate resources of the mucosa (19, 30). Thus, LGG is able to grow with mucin as a carbon source (31) and it possesses extracellular factors that upregulate the production of mucin in the colonic epithelium (32). Some authors have reported that LGG, although being able to use mucus-derived glycans, was not capable of fermenting L-fucose (31). However, results obtained by others (30) and those presented here showed that LGG ferments L-fucose. The explanation for this may derive from differences in isolates of LGG from different laboratories. Thus, it has been reported that LGG isolates from diverse commercial probiotic products present heterogeneity in their genome sequences, which include point mutations and deletions of big portions of the chromosome (33).

In this work we have established that the fuc genes present in the LGG genome are indeed responsible for the observed L-fucose fermenting capacity of this strain. The presence of specific fuc genes, mutant analysis and the fact that 1,2-propanediol was detected in culture supernatants of LGG grown with L-fucose, supports the idea that the utilization of this sugar in LGG follows the same pathway to that described in E. coli. Depending on the growth conditions (anaerobic or aerobic), E. coli directs the lactaldehyde resulting from L-fucose catabolism towards 1,2-propanediol (FucO activity) or lactate (AldA activity) (10). Although the LGG genes responsible for lactaldehyde metabolism have not been identified yet, it is possible that the redox status dictates its
metabolism towards these two compounds in *L. rhamnosus*. However, we showed that L-fucose catabolism in LGG was only favored under anaerobic conditions, which suggests that lactaldehyde conversion to L-lactate is not efficient in this strain.

The utilization of carbohydrate resources typically found in the gastrointestinal niche is a characteristic of members of the intestinal microbiota, such as *Bacteroides* or *Bifidobacterium* but also of intestinal lactobacilli. Consequently, the genomes of species such as *Lactobacillus acidophilus*, *Lactobacillus johnsonii* or *Lactobacillus gasseri* carry genes encoding many carbohydrate catabolic pathways, although they lack sialidases, α-fucosidases or *N*-acetylglucosaminidases which are typical in other intestinal commensals or pathogens (34-36). In this respect, the *L. casei / paracasei / rhamnosus* group present unique features, as they are the only lactobacilli where α-fucosidases and genes for the utilization of L-fucose are present. A recent analysis of 100 *L. rhamnosus* strains isolated from several sources showed that they can be clustered on the basis of their sugar fermenting capacity (30). Strains having an L-fucose fermenting phenotype were derived from the oral and intestinal habitat, whereas strains from dairy origin are L-fucose negative. This is in agreement with the hypothesis that lactobacilli evolved by gene loss and acquisition driven by the particular niches they inhabit. In some cases this resulted in genome reduction after adaptation to less complex environments such as milk (25). Whether the *L. rhamnosus fuc* cluster was present in the ancestor of the *L. casei / paracasei / rhamnosus* species and it was most recently lost in *L. paracasei* and in some strains of *L. rhamnosus* or it represents a recent acquisition remains to be elucidated. Interestingly, the *fuc* locus of LGG is located in a chromosomal region which seems dedicated to the catabolism of L-deoxy-sugars, as it also contains a cluster with genes (*rha*) putatively involved in L-rhamnose catabolism, the sugar from which the species
name is derived. However, previous data and our own results (data not shown) showed that LGG was not able to utilize L-rhamnose (19, 30).

The LGG fuc genes are subject to a dual regulation: induction by growth on L-fucose triggered by FucR and catabolite repression probably dependent on the CcpA transcriptional regulator. In B. thetaiotaomicron L-fucose is the inducer of the fuc genes through its binding to a GntR-superfamily transcriptional repressor (11). Transcriptional analysis in LGG grown with Fuc-α1,3-GlcNAc, which leads to the intracellular generation of L-fucose, excludes the fact that L-fucose itself could be the effector of FucR, as it failed to induce the fuc genes in a mutant deficient in the first step of the catabolic route (fucI). Furthermore, FucP does not participate in the regulation of fuc genes. Genetic studies in E. coli pointed to fuculose-1-phosphate as the inducer molecule of the fuc operon via FucR. The same situation probably exists for L. rhamnosus, as the regulators of both species belong to the same DeoR class. FucR DNA binding sites have not been experimentally established for E. coli or other bacteria. In Enterobacteria a consensus sequence for binding can be derived from the alignment of several fuc promoters, resulting in a 36-bp imperfect inverted repeated sequence. Inspection of the LGG fuc promoters did not reveal similar sequences but two tandem repetitions of the sequence TGAAGAAAA separated by 14 bp are present in the fucI promoter and another similar sequence is present in the fucA-fucR intergenic region. Whether these sequences are the target for FucR has to be verified.

L. casei BL23 and LGG are the only lactobacilli described so far able to use fucosylated oligosaccharides (29, 37). Both strains share the same set of α-L-fucosidases (AlfA, AlfB and AlfC) which can act on specific oligosaccharides derived from glycoconjugates present at mucosal surfaces, such as the Lewis^X antigen core, but also human milk oligosaccharides (37). In addition, the alfRB-EFG operon, responsible for the uptake and
hydrolysis of Fuc-α1,3-GlcNAc, is present in both strains (29). However, BL23 does not possess a pathway for L-fucose and, similar to LGG mutants in fucI and fucR, expels the L-fucose moiety of Fuc-α1,3-GlcNAc. A minimal amount of the L-fucose derived from Fuc-α1,3-GlcNAc was detected in the supernatants of the LGG fucP mutant (3%), while this sugar was totally absent in the supernatants from the wild type under the same conditions. This suggests that part of the intracellularly generated L-fucose is diffusing to the extracellular medium and that in the absence of FucP this released sugar cannot re-enter the cell for being metabolized. In accordance to the activating role of FucR, no induction of the fuc genes was detected in the fucR mutant when grown with Fuc-α1,3-GlcNAc. In this mutant only 30% of the intracellular L-fucose from the disaccharide was utilized, which suggests that the basal transcription of the fuc catabolic genes in the absence of activator allows a limited L-fucose metabolism when this sugar is produced intracellularly.

Intestinal bacteria belonging to Bifidobacterium and Bacteroides are well adapted to exploit the intestinal carbohydrate resources and they possess α-L-fucosidases which allow them to scavenge L-fucose from the mucosa (38, 39). However, intestinal commensals such as E. coli, that do not express α-L-fucosidases, depend on other bacterial groups for L-fucose release from fucose-containing glycans. The LGG fuc genes studied here probably represent an adaptation of this strain to dwell in the particular niche of the gastrointestinal tract. However, as LGG α-L-fucosidases are intracellular enzymes, this strain must probably rely on the fucosidase and glycosidase activities from other members of the intestinal microbiota for the cross-feeding of L-fucose and fucosyl-oligosaccharides.
Acknowledgements

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References


Table 1. Strains and plasmids used in this study

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<th>strain or plasmid</th>
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<tr>
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<td>ATCC®50103</td>
</tr>
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<td>L. rhamnosus BL394</td>
<td>LGG with an 963-bp in-frame deletion in fucP</td>
<td>this work</td>
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<td>LGG with a frameshift at the fucI EcoRI site</td>
<td>this work</td>
</tr>
<tr>
<td>L. rhamnosus BL396</td>
<td>LGG with an insertion of a pRV300-derivative at the fucR gene; EryR&lt;sup&gt;o&lt;/sup&gt;</td>
<td>this work</td>
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<tr>
<td>L. casei ATCC393</td>
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<td>ATCC</td>
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<tr>
<td>L. zeae KCTC3804</td>
<td>wild type; very similar to L. casei isolates</td>
<td>ATCC15820</td>
</tr>
<tr>
<td>E. coli DH10B</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 recA1 galE15 galK16 nupG rpsL Invitrogen D&lt;sup&gt;lacX74&lt;/sup&gt; Φ&lt;sup&gt;80lacZΔM15&lt;/sup&gt; araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRV300</td>
<td>non-replicative plasmid for Lactobacillus. AmpR&lt;sup&gt;o&lt;/sup&gt;, EryR&lt;sup&gt;o&lt;/sup&gt; (20)</td>
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<sup>a</sup>American Type Culture Collection; <sup>b</sup>Erythromycin resistance; <sup>c</sup>Chloramphenicol resistance; <sup>d</sup>Ampicillin resistance
Table 2. Product formation and growth characteristics of LGG with L-fucose or D-glucose under different conditions

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<tr>
<td></td>
<td>L-fucose</td>
<td>D-glucose</td>
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<tr>
<td>1,2-Propanediol$^b$</td>
<td>10.0 ± 0.1</td>
<td>ND$^c$</td>
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<tr>
<td>Lactate$^b$</td>
<td>10.0 ± 1.3</td>
<td>33.8 ± 1.3</td>
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<tr>
<td>Residual sugar$^b$</td>
<td>0.10 ± 0.06</td>
<td>ND</td>
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<tr>
<td>Final OD$_{550nm}$</td>
<td>1.20 ± 0.14</td>
<td>1.75 ± 0.07</td>
</tr>
<tr>
<td>Final pH</td>
<td>5.36 ± 0.01</td>
<td>5.02 ± 0.01</td>
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</tbody>
</table>

$^a$Growth was carried out in basal MRS medium supplemented with 20 mM L-fucose or 20 mM D-glucose for 24 h at 37ºC; $^b$results are in mM; $^c$ND; not detected.
Figure legends

Figure 1. L-fucose metabolism in *L. rhamnosus* GG. (A) Schematic representation of the *fuc* gene cluster in LGG. Stem-loops represent putative rho-independent transcriptional terminators and their calculated ΔG values are given. The genetic structures of the different *fuc* mutants generated are shown; (B) proposed catabolic pathway for L-fucose utilization in LGG. L-lactaldehyde can follow two different routes, leading to the production of L-1,2-propanediol or L-lactate. In LGG the route producing L-1,2-propanediol is more efficient (anaerobic growth; see text).

Figure 2. L-fucose catabolic gene clusters in bacteria. A schematic representation of different gene clusters for L-fucose catabolism is shown. In *S. pneumoniae* strains two different *fuc* clusters (type 1 and type 2) have been described. Genes encoding glycosylhydrolases (GH) are shown with the GH family number.

Figure 3. Growth of *L. rhamnosus* GG on L-fucose. (A) *fucI* mutant; (B) *fucP* mutant; (C) *fucR* mutant. In all graphs the growth profile of the wild-type strain is represented for a better comparison. L-fucose concentration was 0.5% (30 mM). Data presented are the means from three determinations. SD did not exceed 15%.

Figure 4. Expression of *fuc* genes in *L. rhamnosus* GG. LGG wild-type strain was grown with 0.5% L-fucose or 0.5% L-fucose plus 0.2% glucose and expression of *fuc* genes was determined by RT-qPCR. The relative expression is referred to bacterial cells.
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Figure 5. Growth of L. rhamnosus GG with fucosyl-α-1,3-N-acetylglucosamine. (A) fucI mutant; (B) fucP mutant; (C) fucR mutant. In all graphs the growth pattern of the wild-type strain is represented for a better comparison. Fucosyl-α-1,3-N-acetylglucosamine was used at 4 mM. Data presented are the means from three determinations. SD did not exceed 15%.

Figure 6. Expression of fuc genes in L. rhamnosus GG grown in fucosyl-α-1,3-N-acetylglucosamine. LGG wild-type, fucP, fucI and fucR strains were grown in the presence of 4 mM fucosyl-α-1,3-N-acetylglucosamine and expression of fuc genes was monitored by RT-qPCR. For each bacterial strain the relative expression is referred to the expression in the same strain grown with 4 mM glucose. Expression in wild type in the presence of 4 mM L-fucose is also shown. The LGG pyrG (LGG_02546), recG (LGG_01660) and leuS (LGG_00848) genes were used as reference.
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