Molecular analysis of the glucose-specific phosphoenolpyruvate:sugar phosphotransferase system from *Lactobacillus casei* and its links with the control of sugar metabolism

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*Lactobacillus casei* transports glucose preferentially by a mannose-class phosphoenolpyruvate:sugar phosphotransferase system (PTS). The genomic analysis of *L. casei* allowed the authors to find a gene cluster (*manLMNO*) encoding the IIAB (*manL*), IIC (*manM*) and IID (*manN*) proteins of a mannose-class PTS, and a putative 121 aa protein of unknown function (encoded by *manO*), homologues of which are also present in *man* clusters that encode glucose/mannose transporters in other Gram-positive bacteria. The *L. casei* man operon is constitutively expressed into a *manLMNO* messenger, but an additional *manO* transcript was also detected. Upstream of the man operon, two genes (*upsR* and *upsA*) were found which encode proteins resembling a transcriptional regulator and a membrane protein, respectively. Disruption of either *upsR* or *upsA* did not affect *manLMNO* transcription, and had no effect on glucose uptake. Cells carrying a *manO* deletion transported glucose at a rate similar to that of the wild-type strain. By contrast, a *manM* disruption resulted in cells unable to transport glucose by the PTS, thus confirming the functional role of the *man* genes. In addition, the *manM* mutant exhibited neither inducer exclusion of maltose nor glucose repression. This result confirms the need for glucose transport through the PTS to trigger these regulatory processes in *L. casei*.

INTRODUCTION

*Lactobacillus casei* is a facultative heterofermentative lactic acid bacterium frequently used as a cheese starter culture and which is also employed as a probiotic. *L. casei* has developed a great efficiency in the utilization of carbohydrates by the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (Viana et al., 2000), and this is supported by the large number of genes encoding PTS proteins that are present in its genome (Klaenhammer et al., 2002). The PTS consists of the general phosphotransferase proteins, enzyme I (EI) and HPr, and carbohydrate-specific transporters. Together, they constitute a phosphorylation cascade that starts at EI, which can be autophosphorylated at a histidine residue by PEP. Phospho-EI transfers the phosphoryl group to HPr, which becomes phosphorylated at the conserved histidine-15 residue. P~His-HPr functions as a phosphoryl donor to the different PTS transporters, which consist of three different proteins or domains (IIA, IIB and IIC) (Postma et al., 1993). In some bacteria, the main glucose PTS transporter is a member of the mannose-class PTSs. Unlike other PTS transporters, mannose-class PTSs are composed of four independent proteins or domains: two cytoplasmic (IIA and IIB) domains, and an additional membrane domain (IID) which forms the permease together with IIC (Postma et al., 1993). The cytoplasmic components use a conserved histidine residue for the phosphoryl transfer, while in the IIB subunits of other PTS families, this function is carried out by a cysteine residue (Charrier et al., 1997; Erni et al., 1989; Postma et al., 1993). Mannose/glucose transporters (PTS<sub>Man</sub>) constitute a distinct group within mannose-class PTS transporters and characteristically possess fused IIA and IIB domains (Zúñiga et al., 2005). The biochemical characterization of some of these transporters has revealed that they have a relatively broad substrate specificity, being able to transport mannose,
glucose, N-acetylglucosamine, fructose and 2-deoxyglucose (2-DG).

In addition to its role in sugar transport, the PTS is implicated in several regulatory processes. P~His-HPr, the form involved in sugar transport through the PTS, can also transfer its phosphate to other non-PTS proteins, thereby regulating their functions (Deutscher et al., 2002; Stülke et al., 1998). Furthermore, HPr can be phosphorylated at residue serine-46 by an ATP-dependent metabolite-activated protein kinase (HPrK/P) (Galinier et al., 1998; Kravanja et al., 1999; Reizer et al., 1998). P-Ser-HPr participates in the mechanism of sugar-uptake regulation known as inducer exclusion and in carbon catabolite repression (CCR), acting as a cofactor of the transcriptional regulator CcpA (Deutscher et al., 1995; Schumacher et al., 2004).

Early biochemical and physiological studies, which were based on the isolation of mutant strains resistant to the toxic glucose analogue 2-DG, allowed the identification of PTS\textsuperscript{\text{Man}} as the main glucose transporter in many lactic acid bacteria (LAB) (Abe & Uchida, 1989; Gauthier et al., 1989; Thompson & Chassy, 1985; Veyrat et al., 1994). In addition to its role in glucose uptake, several functions have been assigned to PTS\textsuperscript{\text{Man}} proteins. Mutant strains resistant to 2-DG (affected in PTS\textsuperscript{\text{Man}}) show different pleiotropic effects: they are defective in CCR and have an altered pattern of expression of several enzymes. In particular, Streptococcus salivarius and Streptococcus mutans strains lacking the IIAB\textsuperscript{\text{Man}} component show alterations in the pattern of synthesized proteins, as revealed by two-dimensional electrophoresis. Moreover, in these strains, the expression of certain virulence genes is also diminished (Abranches et al., 2003; Lapointe et al., 1993). Finally, synthesis of the II\textsubscript{C}\textsuperscript{\text{Man}} component of Listeria monocytogenes (\textit{mptC} gene) enhances sensitivity to class Ila bacteriocins (Ramnath et al., 2004).

Gene clusters encoding PTS\textsuperscript{\text{Man}} proteins have been characterized in many firmicutes, including \textit{S. salivarius} (Lortie et al., 2000), \textit{S. mutans} (Abranches et al., 2003), Streptococcus thermophilus (Cochu et al., 2003), Streptococcus bovis (Asanuma et al., 2004), Enterococcus faecalis (Héchard et al., 2001) and \textit{List. monocytogenes} (Dalet et al., 2001). In this work, we have identified the \textit{man} operon from \textit{L. casei}. In order to study its involvement in the regulation of sugar metabolism, several genes of the \textit{man} operon were disrupted or deleted, and the effect of these mutations on sugar uptake and CCR was investigated.

**METHODS**

**Bacterial strains, growth conditions and plasmids.** The strains and plasmids used in this study are listed in Table 1. The \textit{L. casei} strains were grown at 37 °C under static conditions on MRS medium (Oxoid), MRS basal medium (Veyrat et al., 1994) or MRS fermentation medium (Ada-Micro), supplemented with 0.5-5% of the appropriate sugar, as indicated. Sensitivity to the glucose analogue 2-DG was assayed in MRS fermentation medium supplemented with 0.5-5% lactose as carbon source and 10 mM 2-DG. \textit{Escherichia coli} DH5\textsubscript{x} was used as host in cloning experiments and was grown in Luria–Bertani medium at 37 °C with vigorous shaking. Agar plates containing the same media were prepared by adding 1-5% agar. \textit{E. coli} and \textit{L. casei} transformants were selected with ampicillin (50 \textmu g ml\textsuperscript{-1}) and erythromycin (5 \textmu g ml\textsuperscript{-1}), respectively.

**Table 1. Abbreviations: Strains and plasmids used in this study**

Abbreviations: Amp\textsuperscript{R}, ampicillin resistance; CECT, Spanish Collection of Type Cultures; Erm\textsuperscript{R}, erythromycin resistance; MCS, multiple cloning site.

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<tr>
<th>Strain or plasmid</th>
<th>Genotype or properties</th>
<th>Source</th>
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<tr>
<td><strong>Lactobacillus casei</strong></td>
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<tr>
<td>BL23</td>
<td>Wild-type</td>
<td>CECT 5275</td>
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<td>BL30</td>
<td>Spontaneous 2-DG\textsuperscript{R} (\textit{manL})</td>
<td>Veyrat \textit{et al.} (1994)</td>
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<td>BL240</td>
<td>\textit{manM}::pRV\textit{manM1}</td>
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<tr>
<td>BL242</td>
<td>\textit{upsR}::pRV\textit{upsR1}</td>
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<td>BL243</td>
<td>\textit{ΔmanO}</td>
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<td>DH5\textsubscript{x}</td>
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<td>\textit{thi-1 recA1 relA1 supE44 ΔlacU169 (\Phi80 lacZ ΔM15)}</td>
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<td>Amp\textsuperscript{R}</td>
<td>Invitrogen</td>
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<td>pRV300</td>
<td>Suicide vector carrying Erm\textsuperscript{R} from \textit{pAMβ1} MCS flanked by T7 and T3 promoters</td>
<td>Leloup \textit{et al.} (1997)</td>
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<td>pRV\textit{manM1}</td>
<td>pRV300 with an internal \textit{manM} fragment</td>
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<td>pRV\textit{upsA1}</td>
<td>pRV300 with an internal \textit{upsA} fragment</td>
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<tr>
<td>pRV\textit{upsR1}</td>
<td>pRV300 with an internal \textit{upsR} fragment</td>
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<tr>
<td>pRV\textit{manNO}</td>
<td>pRV300 with a fragment carrying a 5’ deletion in \textit{manO}</td>
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The *L. casei* DNA library, which served for genome sequencing, was constructed in pCDNA2.1 (A. Mazé, A. Hartke & J. Deutscher, unpublished results). Vector pRV300 (Leloup et al., 1997) was used for cloning purposes in *E. coli* and for insertional inactivation of genes in *L. casei*. *E. coli* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories), as recommended by the manufacturer, and *L. casei* strains were transformed as described elsewhere (Posno et al., 1991).

**DNA manipulation and sequencing.** Total DNA was isolated from *L. casei* as described elsewhere (Posno et al., 1991). Recombinant DNA techniques were performed by following standard procedures (Sambrook et al., 1989). DNA sequencing was carried out by using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase and an automated ABI 310 DNA sequencer (Applied Biosystems). M13 universal and reverse primers, or custom primers hybridizing within the cloned fragments, were used to sequence both strands of DNA. Sequence alignment and detection of putative ORFs were carried out with DNAMAN 4.0 for Windows (Lynnon BioSoft). Sequence similarities were analysed with the BLAST program.

**Construction of upsA, upsR, manM and manO mutant strains.** Internal DNA fragments of *manM*, *upsA* and *upsR* were obtained by PCR using *L. casei* chromosomal DNA. A *manM* fragment was obtained with the primers C221-1 (5'-GGCCAATTTGGAATGATTGG-3') and C221-2 (5'-TGTTAATACCATGGCAATC-3'); the *upsA* fragment with primers upsA1 (5'-CATGGTTTGGAGCCTGCC-3') and upsA2 (5'-CCATCAGATATGCTGTCAC-3'), and the *upsR* fragment with primers manR1 (5'-TAAACCATTTGCTGATCCG-3') and manR2 (5'-GACTCAGGCCGCTTGGT-3'). These DNA fragments were cloned in the integrative vector pRV300 digested with EcoRI, giving pRVmanM1, pRVupsA1 and pRV-upsR1, respectively, and they were used to transform the *L. casei* wild-type strain BL23. Chromosomal integration at the correct location was confirmed by PCR analysis and DNA sequencing. Subsequently, one integrant of each type was selected for further studies and these were named BL240, BL241 and BL242, respectively. For the construction of a *manO*-deleted strain, the manO*3'* end was amplified with oligonucleotides D221-2 (5'-ACGCCAAGT-IACCTGG-3') and manO1 (5'-TAAACTCATTGGAAGC-3'), and the 3' end of *manO* was amplified with manO2 (5'-CGTTGG-AATGAGATTTGGG-3') and manO3 (5'-CGTCTAATTGACCAGTGG-3'). The two PCR products were digested with EcoRI and EcoRI/HindIII, respectively, and cloned in one step into Smal/HindIII-digested pRV300, giving pRVmanNO. This plasmid was used to transform the *L. casei* wild-type strain, and single cross-over integrants were selected as erythromycin-resistant clones. One of these integrants was grown for 200 generations in the absence of antibiotic, and clones which underwent a second recombination event were selected as erythromycin-sensitive colonies. In one of these clones, which was named BL243, the deletion of the 5' portion of *manO* was confirmed by PCR analysis.

**RNA isolation and Northern blot analysis.** RNA was isolated from *L. casei* cells grown to an OD$_{530}$ of 0.8 in MRS fermentation medium containing 0.5% of the appropriate sugars. Cells were collected by centrifugation, washed with 50 mM EDTA, pH 8.0, resuspended in 1 ml Tris/EDTA (Gibco-BRL) and mechanically disrupted in a Mini-Beadbeater (Biospec Products). Total RNA was isolated according to the protocol of the Trizol manufacturer. Sample preparation, denaturing agarose gel electrophoresis and RNA transfer were performed by standard methods (Sambrook et al., 1989). The RNA probe for the *manM* gene was made by using plasmid pRVmanM1, which carries an internal *manM* fragment, as template for the in vitro synthesis of the antisense RNA, which was carried out with T7 RNA polymerase and the reagents from the Roche digoxigenin-RNA labelling kit. A *manO* probe was obtained from EcoRI-digested pRVmanNO and T7 RNA polymerase. Hybridization, washing and staining were performed as recommended by the supplier using the CDP-Star chemiluminescent substrate (Roche).

**Determination of transcriptional start sites and RT-PCR.** The transcription initiation of *manL* was determined with the 5'3' RACE (rapid amplification of cDNA ends) kit (Roche), following the manufacturer's instructions. Reverse transcription reactions were performed with total RNA isolated from BL23 grown on glucose and with primer A112-1 (5'-TCTTGATACCTTCGATCC-3'). The cDNA was dA-tailed, and then amplified by PCR using the primers oligo d'T-anchor supplied in the kit and A112-1. The resulting PCR product was used in a second PCR with primers PCR-anchor (supplied with the kit) and A112-2 (5'-ACTCTTACGTCTGGAGCC-3'). The amplified DNA fragment of about 0.3 kb was purified and sequenced. Determination of the *manO* transcriptional initiation site was determined with the same kit by using the oligonucleotide manO4 (5'-CTAAACGGCCGTTTGATAC-3') for cDNA synthesis. The 0.35 kb PCR product was purified and sequenced. RT-PCR was carried out to verify if *manL* and *manO* genes were cotranscribed. RNA samples from *L. casei* wild-type isolated from glucose-grown cells were treated with amplification grade deoxyribonuclease I (Sigma) to eliminate contaminating DNA. Reverse transcription reactions were performed with 5 μg total RNA using avian myeloblastosis virus reverse transcriptase (AMV-RT) (Sigma) with oligonucleotide manO5 (5'-AGATACCGGCAATCTGG-3'). The reverse transcription reactions (20 μl) were carried out as recommended by the manufacturer. The subsequent PCR amplifications were performed with 5 μl of each RT reaction mixture with oligonucleotides manO5 and D221-2 described above.

**Sugar uptake and inducer-exclusion experiments.** D-[14C]Glucose uptake by whole cells of *L. casei* was performed according to Chassy & Thompson (1983a, b). BL23, BL240, BL241, BL242 and BL243 were grown in 50 ml MRS fermentation medium supplemented with 0.5% glucose or 0.5% fructose to an OD$_{530}$ of 0.8. Cells were collected by centrifugation, washed twice with 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM MgCl$_2$, and resuspended in the same buffer [0.1 mg (dry weight) ml$^{-1}$]. Cells were incubated at 37 °C, and 0.5 mM D-glucose (125 μM final concentration, 0-6 mCi mmol$^{-1}$, 22 MBq mmol$^{-1}$) was added. At intervals of 0, 0-25, 0-5, 1, 2, 4 and 8 min, samples (1 ml) were withdrawn and filtered through 0.2 μm pore-size Millipore membranes. Filters were washed and radioactivity was quantified by scintillation counting. Inducer exclusion of D-[14C]maltose transport (0.5 mM final concentration, 0-5 mCi mmol$^{-1}$, 19 MBq mmol$^{-1}$) by glucose was carried out with maltose-grown cells, as described previously (Viana et al., 2000).

**Enzymic activities.** N-Acetylglucosaminidase activity was measured in permeabilized *L. casei* cells. For permeabilization, cells were collected as for RNA isolation, washed with 10 ml 50 mM potassium phosphate buffer, pH 6-8, and resuspended in a one-tenth volume of the same buffer. Fifty microlitres of toluene:acetone (1:9) was added and cells were vortexed at full speed for 5 min. Ten microlitres of the cell suspension was added to 250 μl 10 mM potassium phosphate buffer, pH 6-8, 1 mM MgCl$_2$, 5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma), and the mixture was incubated at 37 °C for 10 min. The reaction was stopped with 250 μl 5% Na$_2$CO$_3$, and the absorbance was measured at 420 nm.

To determine l-sorbose-1-phosphate reductase activity, *L. casei* strains were grown to an OD$_{530}$ of 0-8 in MRS fermentation medium containing 0.5% of the appropriate sugars. Crude extracts were obtained by shaking the cells in a Mini-Beadbeater with 0.1 mm glass beads (four cycles of 30 s at maximal speed with intervals of 1 min on ice). Protein concentrations were determined with the Bio-Rad

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dye-binding assay. L-Sorbose-1-phosphate reductase activity was determined as described by Yebra et al. (2000). Fructose-1-phosphate (2-5 mM) was used as the substrate. The reaction mixture (0-5 ml) contained 12.5 mM MOPS buffer, pH 6.2, 0.2 mM MnCl2, 0.1 mM NADH and 10 lM crude extract. The rate of NADH oxidation was determined by measuring the rate of absorbance change at 340 nm.

Specific enzymic activity is given in nanomoles per minute per milligram of protein.

Determination of the phosphorylation state of HPr. L. casei cells were grown in MRS medium (50 ml) containing 0.5% glucose to an OD540 of 0.6. The pH of the culture was lowered to 4.5 by adding concentrated HCl. Cells were collected by centrifugation at 4 °C, resuspended in 1 ml cold 20 mM potassium acetate buffer, pH 4.5, and broken in a Mini-Beadbeater using glass beads. Cell-free extracts were obtained by centrifugation for 10 min at 14,000 g and 4 °C and were separated on non-denaturing 15% polyacrylamide gels, which allowed the separation of phosphorylated and non-phosphorylated HPr. Proteins were electroblotted onto nitrocellulose membranes, and HPr was detected using a rabbit antiserum raised against Bacillus subtilis HPr as first antibody and anti-rabbit IgG conjugated to alkaline phosphatase as second antibody.

RESULTS

Identification of the PTSMan in L. casei: 2-DG sensibility is abolished in a manM mutant

Previous studies had shown that glucose uptake by the PTS is involved in the regulation of the ribose, lactose and sorbose catabolic pathways in L. casei (Veyrat et al., 1994; Yebra et al., 2000). In order to identify the genes encoding the PTSMan in L. casei, a search for genes encoding putative IID proteins was performed in the draft genomic sequence of L. casei BL23 (Klaenhammer et al., 2002). In addition to IIDsof, IIDlev and IIDeau already described in L. casei (Yebra et al., 2000, 2004; Mazé et al., 2004) we found 11 ORFs encoding putative IID proteins. An alignment of these proteins showed that one of them (manN) displays an additional 33 aa extension at its C terminus (data not shown), which is characteristic of mannose/glucose PTS transporters of firmicutes (Lortie et al., 2000; Dalet et al., 2001). This putative protein is encoded by the distal gene of a gene cluster that also includes the manL and manM genes, which are all present in a 6460 bp DNA fragment (Fig. 1a). Gene manL encodes a putative IIAB fusion protein which shows significant similarity to the IIA and IIB domains of the mannose-class PTS transporters, while manM encodes a protein significantly similar to IIC domains. The putative histidine phosphorylation sites found in other IIA and IIB proteins of the mannose class are also present in the IIAB protein encoded by manL. (LASHGQV for IIA and RLLHGQV for IIB, putative phosphorylation sites underlined). Furthermore, an additional gene (manO) is located downstream of manN. This gene would encode a small (121 aa) basic protein of unknown function (DUF956 family), which is only present in man operons encoding mannose/glucose transporters of Gram-positive organisms (Zuñiga et al., 2005). In conclusion, the presence of manO, the fusion of the IIA and IIB domains, and the extension of IID, all strongly suggested that this operon encodes a mannose/glucose-specific PTS. A phylogenetic reconstruction also showed that these domains are closely related to other mannose/glucose transporters (unpublished results). Two putative rho-independent terminators are located in the manN 5’ region, one between manN and manO (ΔG =−11.1 kcal mol−1, −46.4 kJ mol−1) and another downstream of manO (ΔG =−21.4 kcal mol−1, −89.5 kJ mol−1). Upstream of manL, two ORFs, upsA and upsR (ups for upstream), were present (Fig. 1a). The deduced amino acid sequence of upsA showed significant similarity to a family of bacterial proteins of unknown function (DUF894), many of which are annotated as putative transmembrane transport proteins. upsR showed significant homology to transcriptional regulators, having the highest similarity (25% identity) to a putative positive regulator from Streptococcus pyogenes (GenBank accession no. NP_268807).

To verify that these genes encode a functional glucose PTS transporter, an L. casei manM mutant (BL240) was constructed and analysed for its ability to grow on MRS fermentation medium supplemented with lactose plus 2-DG. The manM mutant was able to grow in media containing 2-DG, indicating that the IICMan domain was necessary for the uptake of this toxic sugar. In order to determine whether upsA and upsR would play a role in glucose transport, upsA (BL241) and upsR (BL242) mutant strains were constructed (Table 1). Neither the upsA nor the upsR mutant could grow on 2-DG-containing medium, suggesting that the inactivation of those genes did not interrupt 2-DG transport by L. casei cells.

L. casei BL30 has previously been isolated as a spontaneous mutant resistant to 2-DG and affected in PTSMan activity (Veyrat et al., 1994). In order to identify the mutation present in this strain, its manLMNO genes were sequenced. A 16 bp deletion was found in manL at the level of a 6 bp direct repeat, which introduced a frameshift in manL (Fig. 2). This implied the loss of the region encoding the putative IIB phosphorylatable histidine, which explains the observed phenotype.

Transcriptional analysis of the man operon

Northern blot experiments were performed with a manM-specific probe and RNA isolated from BL23, BL241 (upsA mutant) and BL242 (upsR mutant) grown in media containing glucose, mannose, fructose or ribose (Fig. 3a). An mRNA band of approximately 3.5 kb was detected, which is in agreement with the expected size (3543 bp) of the DNA region comprising the manL, manM, manN and manO genes and the stem–loop structure located after the manO stop codon (Fig. 1a). This evidence was further supported by RT-PCR analysis, which demonstrated that manO was cotranscribed with manLMN (Fig. 3b). These data imply that the possible rho-independent terminator located downstream of manN is not fully functional. The transcriptional initiation site was determined by 5’-RACE and shown to be located at 81 (G) or possibly 82 (T) bp (due to a possible artefact of RACE when the start is a T) upstream of the manL.
A classical vegetative promoter was found immediately upstream of the transcriptional initiation site (Fig. 1b). Similar levels of transcripts were detected with the sugar assimilated by the manM-targeted probe for every sugar assayed (Fig. 3a), suggesting that expression of the man operon is not affected by the carbon source. Also, no differences in transcription levels were evident with RNAs isolated from upsA and upsR mutants compared to the wild-type strain, indicating that the expression of the man genes is independent of the upsA and upsR gene products.

When using a manO-specific probe, an additional 400 bp transcript was detected (Fig. 3c). This transcript would cover the whole manO gene, and its levels showed some variation depending on the sugar used for growth, being higher on ribose-grown cells. The determination of the 5' end of this specific messenger showed that it started 2105 (C) or possibly 2106 (T) bp upstream of the manO start codon (Fig. 1c). No sequences matching the 210 or 235 consensus could be clearly detected in that area. Instead, a perfect inverted repeat (DGG27 ? 7 kcal mol1, 2116 kJ mol1) was found.

Glucose uptake in the wild-type and manM mutant

We determined the uptake of glucose in L. casei wild-type and manM mutant strains grown on glucose- or fructose-containing medium (Fig. 4). The manM mutant internalized glucose at a significantly lower rate than the wild-type,
indicating that this mutant was impaired in the main glucose transport system in L. casei, the PTS\textsuperscript{Man}. The remaining glucose uptake in the \textit{manM} mutant could be due to glucose transport through a proton-driven permease, as shown previously for the \textit{L. casei} strain BL30 resistant to 2-DG (Veyrat \textit{et al.}, 1994). Glucose was incorporated at a similar rate by the wild-type when grown on either glucose or fructose, corroborating the constitutive expression of the \textit{man} operon. BL241 (\textit{upsA}) and BL242 (\textit{upsR}) mutants showed glucose uptake rates similar to those determined for the wild-type, indicating that disruption of \textit{upsA} or \textit{upsR} did not affect the transport of glucose by the PTS\textsuperscript{Man} (data not shown).

\textbf{Construction of a \textit{manO} strain}

\textit{manO} homologues have been found to be associated to mannose/glucose PTS transporter-encoding operons in firmicutes, but they are absent in the \textit{man} operons of \textit{\gamma}-proteobacteria (Zúñiga \textit{et al.}, 2005). In order to elucidate its function, an \textit{L. casei} strain carrying a chromosomal deletion which removed part of the 5’ region of \textit{manO}, including the ribosome-binding site, was constructed (strain BL243). This strain was sensitive to the toxic glucose analogue 2-DG, suggesting that the inactivation of \textit{manO} did not affect 2-DG transport through the PTS. Furthermore, measurement of [\textsuperscript{14}C]glucose uptake did not show any significant difference between the wild-type and the mutant strain (Fig. 5). Similarly, no differences in enzymic activities subject to CCR were observed (data not shown). For the growth rate on glucose-containing medium, a doubling time of 81 ± 4 min for the wild-type and 101 ± 8 min for the \textit{manO} strain was determined.

\textbf{A \textit{manM} disruption affects glucose repression and inducer exclusion}

Previous studies had shown that a functional glucose/mannose PTS is necessary for CCR in \textit{L. casei} (Veyrat \textit{et al.}, 1994; Yebra \textit{et al.}, 2000). We measured two enzymic activities which are repressed by glucose. As shown in Table 2, sorbose-1-phosphate reductase activity, encoded by the \textit{sorE} gene in the sorbose operon, was subject to catabolite repression by glucose in wild-type \textit{L. casei}, displaying a repression factor higher than 100 (enzyme activity of sorbose-grown cells versus activity of sorbose + glucose-grown cells). Disruption of \textit{manM} lowered the repression factor to 1·8. In \textit{L. casei}, \textit{N}-acetylglucosaminidase activity is also low in the presence of glucose and higher on non-repressing sugars, such as ribose. In the wild-type, this activity was repressed by glucose by a factor of 12 (activity of ribose-grown cells versus activity of glucose-grown cells). However, in the \textit{manM} mutant, the repression factor was only 1·7.
Inducer exclusion represents one of the many regulatory processes elicited by the presence of glucose. In this process, glucose uptake inhibits the transport of other carbohydrates that are translocated through PTS or non-PTS permeases (Djordjevic et al., 2001; Dossonnet et al., 2000; Monedero et al., 2001). As shown in Fig. 6, the addition of glucose to L. casei cells transporting maltose, which in L. casei is taken up via an ABC-type transporter (V. Monedero & J. Deutscher, unpublished results), totally prevented the transport of this disaccharide. By contrast, glucose had no inhibitory effect on maltose transport in the manM mutant.

**Effect of a manM mutation on the phosphorylation state of HPr**

In L. casei, the HPr protein of the PTS is involved in CCR and inducer exclusion. To carry out these regulatory functions, HPr needs to be phosphorylated at serine-46 (P-Ser-HPr) (Dossonnet et al., 2000; Viana et al., 2000). As shown in Fig. 6, the addition of glucose to L. casei cells transporting maltose, which in L. casei is taken up via an ABC-type transporter (V. Monedero & J. Deutscher, unpublished results), totally prevented the transport of this disaccharide. By contrast, glucose had no inhibitory effect on maltose transport in the manM mutant.

In this work, the man operon of L. casei BL23 has been identified. This operon includes the genes encoding the IIABMan (manL), IICMan (manM) and IIDMan (manN) proteins, which are all essential for glucose transport via the PTS, as well as the manO gene, which encodes a protein of unknown function. The genetic organization of the man operon in L. casei is similar to that in S. salivarius (Lortie et al., 2000), S. thermophilus (Cochu et al., 2003) and S. bovis (Asanuma et al., 2004). In these species, as well as in L. casei, a single transcript that covers the complete operon (manLMNO) is detected. While transcription of the genes encoding PTSMan transporters in streptococci and L. casei does not seem to be regulated by the carbon source, the man operon of List. monocytogenes is induced by the presence of manM mutation, the different phosphorylated forms of HPr were estimated in wild-type and manM strains grown on glucose. Extracts from both strains were analysed on non-denaturating polyacrylamide gels followed by Western blot, a technique which allows the separation of phosphorylated and non-phosphorylated HPr. In this electrophoresis system, P-Ser-HPr and P~His-HPr migrate to nearly identical positions, but they can be distinguished by heating the samples before loading, which leads to hydrolysis of the thermolabile P~His bond. All possible phosphorylated forms, P~His-HPr, P-Ser-HPr and doubly phosphorylated 2P-(His, Ser)-HPr, could be readily detected in L. casei wild-type cells (Fig. 7). In contrast, while substantial amounts of P~His-HPr were present in the manM strain, no P-Ser-HPr or doubly phosphorylated HPr could be detected. Hence, the CCR and inducer-exclusion defects detected in the manM mutant are probably a direct consequence of the low level of phosphorylation of HPr at the regulatory serine-46.

**DISCUSSION**

In this work, the man operon of L. casei BL23 has been identified. This operon includes the genes encoding the IIABMan (manL), IICMan (manM) and IIDMan (manN) proteins, which are all essential for glucose transport via the PTS, as well as the manO gene, which encodes a protein of unknown function. The genetic organization of the man operon in L. casei is similar to that in S. salivarius (Lortie et al., 2000), S. thermophilus (Cochu et al., 2003) and S. bovis (Asanuma et al., 2004). In these species, as well as in L. casei, a single transcript that covers the complete operon (manLMNO) is detected. While transcription of the genes encoding PTSMan transporters in streptococci and L. casei does not seem to be regulated by the carbon source, the man operon of List. monocytogenes is induced by the presence of...
glucose by means of the ManR regulator (Dalet et al., 2001). Disruption in *L. casei* of the gene encoding a putative transcriptional regulator, *upsR*, or the accompanying *upsA* gene had no effect on *man* transcription or glucose uptake, showing that these genes are not related to PTS<sub>Man</sub> regulation. In *L. casei* ATCC334, the same *man* operon structure is conserved (http://genome.jgi-psf.org/draft_microbes/lacca/lacca.home.html), but an insertion element of 1017 bp is located between *upsA* and *manL*. An additional *manO* transcript has been found in *L. casei* BL23, *S. bovis* (Asanuma et al., 2004) and *S. salivarius* (Lortie et al., 2000). A *manO*-specific promoter has been proposed for the streptococci in the intergenic region between *manN* and *manO*. The 5' end of the *L. casei* *manO* transcript is located at the base of a possible stem–loop structure in the RNA, and a consensus promoter sequence is not found. This makes it possible that the *manO* RNA results from processing of the *manLMNO* transcript at an RNase cleavage site determined by the stem–loop. However, the expected 3·1 kb *manLMN* transcript was not detected.

In order to elucidate the function of the *manO* gene product, we obtained, for the first time, a knock-out strain in this gene. No clear phenotype could be determined for this mutant. *manO* homologues are only present in the genomes of firmicutes possessing a PTS<sub>Man</sub>, and are usually associated with the *man* operon, with the exception of *Lactobacillus plantarum* and *S. mutans*, in which it is located 22 kb and

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**Table 2.** Enzymic activities in wild-type *L. casei* and *manM* mutant strains

*L. casei* strains were grown in MRS fermentation medium supplemented with 0·5% glucose (Glu), 0·5% sorbose (Sor), 0·5% ribose (Rib) or 0·5% glucose plus 0·5% sorbose (Glu+Sor). The activity of L-sorbose-1-phosphate reductase is given in nmol min<sup>−1</sup> (mg protein)<sup>−1</sup>; the activity of N-acetylglucosaminidase is given in nmol min<sup>−1</sup> (mg dry weight)<sup>−1</sup>. Data represent mean±standard deviation from at least three experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity of:</th>
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<tr>
<td></td>
<td>1-Sorbose-1-phosphate reductase (nmol min&lt;sup&gt;−1&lt;/sup&gt;)</td>
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<td></td>
<td>Glu</td>
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<td>BL23 (wild-type)</td>
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<tr>
<td>BL240 (manM)</td>
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**Fig. 6.** Inducer exclusion of [*14C*]maltose transport by glucose with maltose-grown cells from wild-type *L. casei* BL23 (closed symbols) and *manM* mutant BL240 (open symbols). Maltose transport is shown in the absence (circles) and presence of glucose (triangles). Data are average values and ranges of two measurements with cells obtained from independent cultures.

**Fig. 7.** Western blot analysis of crude extracts showing the different HPr forms in *L. casei* BL23 (wild-type) and BL240 (manM) grown in MRS with glucose. The HPr protein can be phosphorylated at two different residues: histidine-15 (P~His-HPr) and serine-46 (P-Ser-HPr). On a non-denaturing gel, P~His-HPr and P-Ser-HPr migrate to nearly the same position. However, as only the phosphate bond of P~His-HPr is thermolabile, the two forms of HPr can be distinguished by heating the samples. +, Samples were heated at 70°C for 10 min before loading, which dephosphorylates P~His-HPr; −, samples were loaded without heating. 2P-(His, Ser)-HPr stands for doubly phosphorylated HPr.
3.2 kb away from manN, respectively. Also, in Streptococcus pneumoniae, manO is placed upstream of manL and separated by a 110 kb fragment and, curiously, Lactococcus lactis has two copies of manO separated by a gene of unknown function (Zúñiga et al., 2005). This, together with the fact that L. casei manO RNA levels showed some variation depending on the carbon source, suggests that the manO gene product participates in some regulatory process.

Glucose transport by the PTS has a remarkable influence on cell physiology, as it regulates the expression of many genes encoding proteins involved in the uptake and metabolism of other carbon sources, and controls the activity of transcriptional regulators (Deutscher et al., 2002; Tigemeyer & Hillen, 2002; Vadeboncoeur & Pelletier, 1997). The analysis of a spontaneous 2-DG-resistant mutant (BL30) lacking glucose-specific PTS activity provided the first evidence that the PTS in L. casei is involved in the CCR effect exerted by glucose on the utilization of lactose, ribose and sorbose (Veyrat et al., 1994; Yebra et al., 2000). It has been shown here that the phenotype of this strain is most likely due to a mutation in the IIAB\textsuperscript{Man} protein encoded by manL. We have also shown that the repression mediated by glucose of sorbose-1-phosphate reductase and N-acetylglucosaminidase activities is partially relieved in another mutant impaired in PTS glucose uptake, due to a lack of the IIC\textsuperscript{Man} protein encoded by manM. Additionally, inducer exclusion exerted by glucose on maltose transport is also abolished in this mutant. In L. casei, CCR and inducer exclusion of non-PTS sugars are controlled by the phosphorylation state of HPr at serine-46 (Viana et al., 2000; Dossonnet et al., 2000). We have shown that a manM mutant grown on glucose accumulates a substantial amount of P\textasciitildeHis-HPr. This is conceivable in view of the fact that the phosphotransfer chain was interrupted at the level of IIC\textsuperscript{Man}, and thus no P\textasciitildeHis-HPr was consumed during sugar transport. By contrast, in wild-type cells actively growing on glucose, P\textasciitildeHis-HPr is preferentially utilized for glucose phosphorylation through the PTS, and low steady-state levels of P\textasciitildeHis-HPr are probably maintained. The apparent low phosphorylation of HPr at serine-46 in the manM mutant could be explained by two facts: (i) P\textasciitildeHis-HPr is a poor substrate for HprK/P-catalysed phosphorylation at serine-46 (Deutscher et al., 1984) and (ii) the reduced rate of sugar utilization caused by a defect in PTS transport would not trigger activation of the kinase function of HprK/P (Dossonnet et al., 2000; Kravanja et al., 1999). It seems, therefore, that the pleiotropic effect of a mutation in the PTS\textsuperscript{Man} proteins could, at least partly, be due to diminished phosphorylation of HPr at serine-46.

In summary, the PTS\textsuperscript{Man} of L. casei has been identified and characterized. We have also shown its participation in glucose repression and regulation of sugar uptake by inducer exclusion. At present, we are designing new experiments to study the possible role of the manO gene product in the regulation of sugar transport.

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