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Characterization of the individual glucose uptake systems of *Lactococcus lactis*: mannose-PTS, cellobiose-PTS and the novel GlcU permease

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Summary

According to previous reports, *Lactococcus lactis* imports glucose via two distinct phospho*enol*pyruvate:phosphotransferase systems (mannose-PTS and cellobiose-PTS) and one or more unknown non-PTS permease(s). GlcU was identified as the sole non-PTS permease involved in the transport of glucose. Additionally, the biochemical properties of PTS^{Man}, PTS^{Cel} and GlcU were characterized in double knock-out mutants with glucose uptake restricted to a single system. Transport susceptibility to protonophores indicated that glucose uptake via GlcU is proton-motive force dependent. Competition assays revealed a high specificity of GlcU for glucose. Furthermore, the permease has low affinity for glucose and displays strong preference for the β-anomer as shown by the profiles of consumption of the two glucose anomers studied by ^{13}C-NMR. Similar kinetic properties were found for PTS^{Cel}, while PTS^{Man} is a high-affinity system recognizing equally well the two anomeric forms of glucose. Transcripts of the genes encoding the three transporters are present simultaneously in the parent strain NZ9000 as shown by reverse transcription-PCR. Investigation of the distribution of GlcU homologues among bacteria showed that these proteins are restricted to the low-GC Gram-positive *Firmicutes*. This work completes the identification of the glucose transport systems in *L. lactis* MG1363.

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Introduction

Glucose is abundant in nature, reasonably cheap, and has been the substrate of choice for most bacteria used in biotechnological applications (de Vos and Hugenholtz, 2004; Gosset, 2005; Wendisch *et al*., 2006; Nevoigt, 2008), since it supports high growth rates and biomass yields. Furthermore, glucose is a preferred sugar in many bacteria and known to repress the synthesis of enzymes necessary for the utilization of other carbohydrates via regulatory mechanisms involving glucose-specific phospho*enol*pyruvate (PEP):sugar phosphotransferase system (PTS) components (Stülke and Hillen, 2000; Deutscher *et al*., 2006; Görke and Stülke, 2008; Jahreis *et al*., 2008).

A wealth of data have been gathered on the different metabolic reactions that convert glucose into pyruvate; however, the first event in the metabolism of any external nutrient, i.e. transport, has only attracted modest attention. Transport of glucose across the bacterial cytoplasmic membrane proceeds via ATP-binding cassette (ABC) transporters (primary active transporters), secondary carriers or group translocators (PEP:sugar PTS) (Postma *et al*., 1993; Ehrmann *et al*., 1998; Pao *et al*., 1998; Jack *et al*., 2001; Konings, 2006; Jahreis *et al*., 2008). The presence of multiple glucose uptake systems is a common feature in many organisms. For example, the Gram-negative model bacterium *Escherichia coli* possesses at least a glucose-PTS (PTS^{Glc}), a mannose-PTS (PTS^{Man}), a proton symporter (GalP) and an ABC transporter (Mgl system) to import glucose (Gosset, 2005), and expression of the genes encoding these transporters is influenced by several factors, such as the nature and the concentration of sugar (Death and Ferenci, 1994; Vanderpool and Gottesman, 2004). The low-GC Gram-positive *Bacillus subtilis* internalizes glucose by PTS^{Glc}, PTS^{Man}, and the glucose/mannose-proton symporter GlcP during vegetative growth (Paulsen *et al*., 1998).

Lactococcus lactis is a low-GC Gram-positive coccoid bacterium that is used worldwide as a constituent of

starter cultures in the dairy industry. The glucose-PTS is not present in this bacterium: glucose is transported and concomitantly phosphorylated by mannose and cellobiose PTSs (PTS^{Man} and PTS^{Cel}). Moreover, glucose can also be imported via non-PTS permease(s) and subsequently phosphorylated by glucokinase (Thompson *et al*., 1985; Pool *et al*., 2006). Hitherto, gene(s) coding for active non-PTS permease(s) in *L. lactis* remained elusive. Therefore, we set out to identify the non-PTS transport system(s) and characterize the routes for glucose uptake. In this work, we demonstrate the role of GlcU in the transport of glucose by this organism. Deletion of *glcU* in a PTS-deficient strain abolished growth on glucose, proving that GlcU is the sole non-PTS permease in *L. lactis*. Biochemical features of the individual transporters were determined in double mutant strains with glucose transport restricted to a single system. Kinetic properties were obtained by studying uptake of radiolabelled glucose in whole cells; moreover, *in vivo* ^{13}C-NMR was used to monitor the consumption of α- and β-anomers of glucose as well as the dynamics of intracellular metabolite pools. The expression of the transporter encoding genes in the parent and mutant strains was evaluated by reverse transcription (RT)-PCR analysis.

Results

GlcU is the only non-PTS glucose permease of L. lactis

Lactococcus lactis NZ9000 transports glucose via PTS^{Man} (*ptnABCD*), PTS^{Cel} (*ptcBAC*) and by thus far unknown non-PTS permease(s). Previously, a transcriptome analysis approach proved useful to identify PTS^{Cel} as a glucose transporter (Pool *et al*., 2006). Assuming that the non-PTS system(s) would be expressed at a higher level in a double-PTS mutant (in which PTS^{Man} and PTS^{Cel} are inactivated), mRNA levels in NZ9000 and this transport mutant were compared using DNA microarrays. Genes encoding proteins with high isoelectric points and with putative transmembrane segments were selected among all genes significantly upregulated (data not shown). The gene with the highest fold overexpression in the PTSs-defective strain was *ytgA* (llmg_2145), which is part of a putative operon (*ytgBAH*). However, deletion of this gene cluster in the double-PTS mutant did not affect growth on glucose, suggesting a minor or even no role of *ytgBAH*-encoding proteins in glucose transport. As a second approach, BLASTP searches of the *L. lactis* MG1363 genome sequence were performed using sequence information on functionally characterized glucose non-PTS permeases. Of these, only the *Staphylococcus xylosus* glucose uptake protein (GlcU) (Fiegler *et al*., 1999) showed homology (36% identity) to an *L. lactis* MG1363 protein that is encoded by llmg_2561 and is annotated as GlcU (Wegmann *et al*., 2007). This gene was not significantly upregulated in the DNA microarray analysis of the double-PTS knockout mutant. Nevertheless, to investigate whether llmg_2561 (*glcU*) encodes a functional glucose permease the gene was deleted in a PTS^{Man}/PTS^{Cel}-deficient strain. First, the PTS^{Cel} route was inactivated in NZ9000Δ*ptnABCD* (Pool *et al*., 2006) by deleting the gene encoding its membrane component, *ptcC*: a 1.25 kb fragment 45 bp downstream of the start codon was removed by double-cross-over recombination. Subsequently, llmg_2561 (*glcU*) was inactivated in strain NZ9000Δ*ptnABCD*Δ*ptcC* by removing a 0.97 kb fragment starting 103 bp upstream of the start codon of the gene. The triple mutant was obtained on M17 medium supplemented with galactose (1%).

The ability of the mutants to grow on glucose was investigated. Inactivation of llmg_2561 (*glcU*) in the PTS-deficient background rendered a strain unable to grow on glucose (Fig. 1A). We verified that this phenotype arises from the inability of the triple mutant to transport glucose (Fig. 1B). Expression *in trans* of *glcU* under the control of the nisin promoter in the triple mutant restored growth on glucose (Fig. 1A). Altogether these findings unequivocally show that the protein encoded by llmg_2561, hereafter denominated GlcU, is the sole PTS-independent glucose transporter in *L. lactis*.

Kinetic properties of L. lactis *glucose transport systems*

The kinetic properties of GlcU were investigated in the double mutant NZ9000Δ*ptnABCD*Δ*ptcC*. To characterize the PTS^{Man} and the PTS^{Cel}, *glcU* was deleted in strains NZ9000Δ*ptcC* and NZ9000Δ*ptnABCD*, respectively, resulting in strains NZ9000Δ*ptcC*Δ*glcU* (only PTS^{Man} functional) and NZ9000Δ*ptnABCD*Δ*glcU* (only PTS^{Cel} functional). The kinetic properties of the various glucose transporters were determined from [^{14}C]-glucose uptake experiments using non-linear regression analysis to estimate K_m and V_{max} values.

Glucose transport via GlcU is proton-motive force dependent. The lactococcal non-PTS permease, GlcU, showed a low affinity (K_m, 2.4 mM) and a moderate capacity (39 nmol min^{-1} mg $prot^{-1}$) for glucose uptake in resting cells of NZ9000Δ*ptnABCD*Δ*ptcC*. To investigate the mechanism of transport via GlcU, the protonophores tetrachlorosalicylanilide (TCS) and carbonyl cyanide-m-chlorophenylhydrazone (CCCP) were used. CCCP and TCS inhibited the uptake of [^{14}C]-glucose by 53% and 100% respectively. Furthermore, cells energized with arginine (formation of ATP via the arginine deiminase pathway) prior to glucose addition showed a twofold (81 nmol min^{-1} mg $prot^{-1}$) increase in the transport capac-

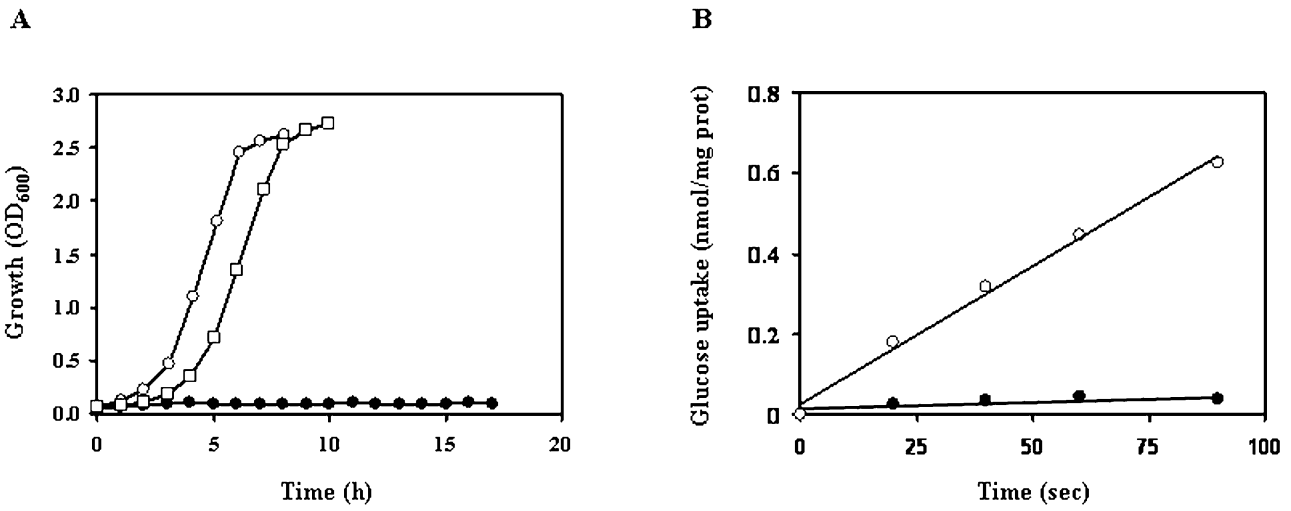

Fig. 1. Identification of *L. lactis* non-PTS permease.
A. Growth in CDM with 1% of glucose (w/v) at 30°C, without pH control (initial pH 6.5).
B. [^{14}C]-glucose uptake in whole cells (KP_i 50 mM, pH 6.5) at 30°C with 10 μM glucose. Nisin (1 ng ml^{-1}) was used to induce expression of *glcU* in the complemented strain.
Symbols: (○) NZ9000Δ*ptnABCD*Δ*ptcC*; (●) NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*; (□) NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*(*glcU*$^+$).

ity (Table 1). Altogether these results indicate that glucose transport via GlcU is driven by the proton-motive force. To further characterize GlcU, transport competition assays were performed in which the uptake of radiolabelled glucose was measured in the presence of 100-fold excess concentration of ribose, rhamnose, mannose, xylose, arabinose, cellobiose and galactose. Glucose uptake was reduced by approximately 30% in the presence of mannose and xylose; the other sugars affected glucose transport to a minor extent (less than 15%).

PTS^{Man} and PTS^{Cel} have different affinity for glucose. Kinetic properties of PTS^{Man} and PTS^{Cel} were determined in whole cells of NZ9000Δ*ptcC*Δ*glcU* and NZ9000Δ*ptnABCD*Δ*glcU*, respectively (Table 1). Both PTS^{Man} and PTS^{Cel} transport glucose with high capacity, but PTS^{Cel} exhibits a much lower affinity (K_m of 8.7 mM compared with 13 μM for PTS^{Man}).

The transport route affects glycolytic flux and dynamics of intracellular metabolites

Lactococcus lactis strains with single glucose uptake systems, PTS^{Man}, PTS^{Cel} or GlcU, were studied by *in vivo* ^{13}C-NMR (Fig. 2). The isogenic *L. lactis* laboratory strains MG1363 and NZ9000 convert glucose homofermentatively with a maximal glucose consumption rate of about 0.40 μmol min^{-1} mg prot^{-1} (Neves *et al.*, 2002a; 2006). All mutants showed lower maximal glucose consumption rates (1.4- to 2.9-fold lower). Glucose consumption in NZ9000Δ*ptcC*Δ*glcU* was quasi-linear up to concentrations close to depletion and the maximal rate was 0.29 μmol min^{-1} mg prot^{-1}, a value similar to that of V_{max} for PTS^{Man} (Table 1). In NZ9000Δ*ptnABCD*Δ*glcU*, glucose consumption was initially relatively high, slowing down (sixfold) at approximately 8 min, at the onset of β-glucose depletion. When uptake of glucose was restricted to GlcU (NZ9000Δ*ptnABCD*Δ*ptcC*) the kinetics of consumption

Table 1. Kinetic parameters for glucose transport via the three different systems.

Strain	Transporter present	V_{max}[a] (μmol min^{-1} mg prot^{-1})	K_m[a] (mM)
NZ9000Δ*ptcC*Δ*glcU*	PTS^{Man}	0.22 ± 0.01	(13 ± 2) × 10^{-3}
NZ9000Δ*ptnABCD*Δ*glcU*	PTS^{Cel}	0.25 ± 0.02	8.7 ± 1.5
NZ9000Δ*ptnABCD*Δ*ptcC*[b]	GlcU	0.08 ± 0.01	2.4 ± 0.2

a. Values of two or more independent experiments were averaged and are reported ±SD. V_{max} and K_m were determined using glucose concentrations varying as follows: NZ9000Δ*ptcC*Δ*glcU*, 1–100 μM; NZ9000Δ*ptnABCD*Δ*glcU*, 0.5–15 mM; NZ9000Δ*ptnABCD*Δ*ptcC*, 0.01–10 mM.
b. Transport determinations were performed in the presence of 2 mM arginine.

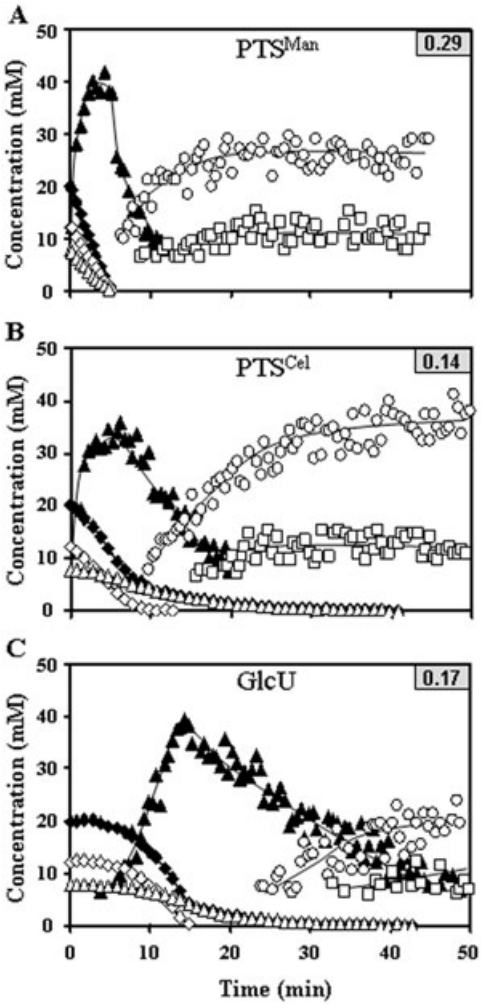

Fig. 2. Glucose metabolism in non-growing cell suspensions of *L. lactis* strains carrying only one active glucose uptake system. Kinetics of [1-^{13}C]-glucose (20 mM) consumption and pools of intracellular metabolites in resting cells of *L. lactis* strains NZ9000Δ*ptcC*Δ*glcU* (A), NZ9000Δ*ptnABCD*Δ*glcU* (B) and NZ9000Δ*ptnABCD*Δ*ptcC* (C), which possess as glucose transporter PTS^{Man} or PTS^{Cel} or GlcU, respectively, at 30°C under anaerobic conditions with pH controlled at 6.5. Maximal glucose consumption rates (μmol min^{-1} mg prot^{-1}) are boxed in the upper-right corner. Symbols: (◆) glucose; (◇) β-glucose; (△) α-glucose; (▲) fructose 1,6-bisphosphate; (○) 3-phosphoglycerate; (□) phospho*enol*pyruvate. The lines drawn in the graph are interpolations. For the sake of simplicity, glucose transients are defined by every 1 min data points in (B) and (C).

was complex (Fig. 2C): an initial lag-phase was followed by a period of acceleration, with the rate varying up to a maximum of 0.17 μmol min^{-1} mg prot^{-1}. Depletion of β-glucose resulted in a drastic reduction of the glucose consumption rate. Energization of cells with arginine (2 mM) prior to glucose addition reduced the lag-time about 2.2-fold and increased the maximal glucose consumption rate to 0.23 μmol min^{-1} mg prot^{-1}, indicating that glucose transport via GlcU is dependent on the energetic status of the cell.

The profile of intracellular metabolites in NZ9000Δ*ptcC*Δ*glcU* (active PTS^{Man}) resembles that of the parent strains (Neves *et al.*, 2002a; 2006), except for the maximal concentrations of 3-phosphoglycerate (3-PGA) and PEP, which were approximately three times higher in the mutant (Fig. 2A). When glucose was internalized via the PTS^{Cel} (in NZ9000Δ*pnABCD*Δ*glcU*) fructose 1,6-bisphosphate (FBP) accumulated rapidly to a concentration of 35 ± 1 mM, subsequently declining to undetectable levels as glucose consumption decreased. Concomitantly, the pools of 3-PGA and PEP rose to maximal concentrations of 35 ± 3 mM and 12 ± 2 mM respectively (Fig. 2B). Curiously, in NZ9000Δ*ptnABCD*Δ*ptcC* (active GlcU) the FBP pool decreased slowly to undetectable values (Fig. 2C); the accumulation of 3-PGA and PEP was delayed as compared with NZ9000Δ*pnABCD*Δ*glcU*, and the PEP potential was about 1.8-fold lower.

Modelling of glucose consumption

The kinetics of α- and β-glucose consumption via the individual glucose transport systems was monitored by *in vivo* ^{13}C-NMR. The model was developed to quantify the α- and β-anomer uptake fluxes and the flux of anomerization using the NMR time-courses for glucose utilization as input data (see Fig. S1). The estimated kinetic parameters are shown in Table 2. Glucose consumption in strain NZ9000Δ*ptnABCD*Δ*ptcC* (active GlcU) was strongly dependent on the energy *status* of the cells (see above); therefore, all calculations were performed using data obtained with energized cells.

Table 2. Kinetic parameters obtained from the profiles of glucose consumption in the parental strain *L. lactis* NZ9000 and its derivatives.

Strain	Transport system	V_{max} (μmol min^{-1} mg prot^{-1})	K_{af}^{α} (mM)	K_{af}^{β} (mM)
Δ*ptcC*Δ*glcU*	PTS^{Man}	0.32 ± 0.01	0.7 ± 0.1	0.8 ± 0.1
Δ*ptnABCD*Δ*glcU*	PTS^{Cel}	0.15 ± 0.01	442 ± 42	0.9 ± 0.1
Δ*ptnABCD*Δ*ptcC*[a]	GlcU	0.53 ± 0.13	122 ± 32	2.6 ± 0.7
NZ9000	PTS^{Man}	0.25 ± 0.01	*0.7*[b]	*0.8*[b]
	<PTS^{Cel}+GlcU>	0.31 ± 0.07	119 ± 43	2.8 ± 1.5

a. The model was fitted to data obtained during the metabolism of glucose in cells energized with arginine (2 mM).
b. The K_{af} values obtained for PTS^{Man} were used to model glucose consumption by *L. lactis* NZ9000.

PTS^{Man} showed no preference for α-glucose or β-glucose, since the two anomers were consumed efficiently (Fig. 2A) and similar affinity constants (K_{af}s) were obtained for the two anomers in strain NZ9000Δ*ptcC*Δ*glcU* (Table 2). Accordingly, the net flux for the anomeric conversion was negligible, indicating that the equilibrium anomeric ratio was maintained during glucose consumption. On the other hand, the very high K_{af} values for α-glucose, the low fluxes of α-glucose consumption, and the low K_{af} values for β-glucose in strains NZ9000Δ*ptnABCD*Δ*glcU* and NZ9000Δ*ptnABCD*Δ*ptcC* denote the strong preference of PTS^{Cel} and GlcU for β-glucose. Therefore, the double-phase kinetics observed for glucose consumption in the latter two strains (Fig. 2) are explained by the inability of PTS^{Cel} and GlcU to import α-glucose; the initial phase corresponds to the fast uptake of the preferred anomer (β-glucose), while the glucose consumption rate in the second phase is limited by the rate of conversion of α- into β-glucose.

Taking advantage of the distinct anomeric specificities of the individual transporters it was possible to characterize glucose consumption in the parental strain *L. lactis* NZ9000. PTS^{Cel} and GlcU were lumped since they displayed similar anomer selectivity (high and low K_{af} values for α- and β-glucose respectively). Therefore, the profile of glucose consumption in strain NZ9000 was modelled with two transport systems: PTS^{Man} and <PTS^{Cel}+GlcU>. Moreover, the K_{af} values determined in the mutant strain NZ9000Δ*ptcC*Δ*glcU* for α- and β-glucose (Table 2) were used to model PTS^{Man} in the parent strain. The calculations indicate that the parent strain takes up α-glucose exclusively via PTS^{Man} (Fig. S1); furthermore, glucose is taken up with similar efficiency by PTS^{Man} and <PTS^{Cel}+GlcU> (similar V_{max} values, see Table 2).

At this stage it should be pointed out that the kinetic parameters obtained by modelling glucose consumption (Table 2) are inherently different from the kinetic parameters obtained from modelling the results of the radiolabelling assays (Table 1). The latter were obtained from assays in which metabolism of glucose was minimized (up to 10 s), while the profiles of glucose consumption were obtained in cells actively metabolizing glucose using *in vivo* NMR, a technique that allows distinguishing the individual anomers. Transport assays were fitted with a Michaelis–Menten function and the consumption of the glucose anomers was fitted with a similar formalism that considered the two anomers as competitors for the same binding site of the transporters. Thus, although K_m and K_{af} represent conceptually the same property it is not surprising that the two models yield different values.

It is also pertinent to stress that the K_ms obtained for PTS^{Cel} and GlcU in the strains NZ9000Δ*ptnABCD*Δ*glcU* and NZ9000Δ*ptnABCD*Δ*ptcC* from the radiolabelling assays (Table 1) are overestimated since these transporters recognize β-glucose only, while total glucose (α plus β) was used to extract the kinetic parameters.

The three transport systems are expressed in the parent strain NZ9000

The level of expression of *glcU*, *ptnC* and *ptcC* in strain NZ9000 and derivatives with a single transporter was investigated by RT-PCR analysis (Fig. 3). The results show that all three genes are transcribed in NZ9000 (Fig. 3A). The expression level of *glcU* and *ptnC* in the double transporter mutants NZ9000Δ*ptnABCD*Δ*ptcC* and NZ9000Δ*ptcC*Δ*glcU*, respectively, was higher than that in the parent strain NZ9000 (Fig. 3B). In contrast, *ptcC* expression was reduced in strain NZ9000Δ*ptnABCD*Δ*glcU* when compared with that in strain NZ9000.

Transcription of *glcU*, *ptnC* and *ptcC* was studied in NZ9000 for the sugars shown to sustain growth in CDM (Fig. 4). The three genes were transcribed under all the conditions tested. Glucose and mannose induced the expression of *glcU*, whereas *ptnC* expression was induced by mannose, cellobiose and maltose. Glucose, cellobiose and maltose stimulated the transcription of *ptcC*.

Discussion

This work completes the identification of the glucose transport systems in *L. lactis* MG1363. Glucose uptake can proceed via PTS^{Man}, PTS^{Cel} and the secondary carrier GlcU, herein disclosed for the first time. Noteworthy, the genes encoding the three different transporters are also present in the genome sequences of *L. lactis* strains IL1403 and SK11 (Bolotin *et al.*, 2001; Makarova *et al.*, 2006). Moreover, it is shown that GlcU is the sole non-PTS permease involved in the transport of glucose in *L. lactis* MG1363. The protein shares 36% identity with the functionally characterized *S. xylosus* glucose uptake protein (Fiegler *et al.*, 1999). The latter is a member of the exclusively prokaryotic glucose/ribose porter family, which belongs to the drug metabolite superfamily of transporters (Jack *et al.*, 2001). A recent genomic analysis revealed that the glucose/ribose porters are well represented in low-GC Gram-positive organisms (Lorca *et al.*, 2007). Moreover, BLASTP searches of all available prokaryotic genome sequences (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) using the lactococcal or the staphylococcal GlcU sequences as queries retrieved homologues only within the low-GC Gram-positive *Firmicutes* (data not shown). In fact, GlcU-like proteins are widespread in the orders *Bacillales* and *Lactobacillales* and are also found in two *Clostridium* spp. strains. In *Bacillus* spp., *glcU* is transcribed during the sporulation process in the forespore (Fujita *et al.*, 1977; Nakatani *et al.*, 1989), whereas vegetative cells express a different non-PTS permease, the

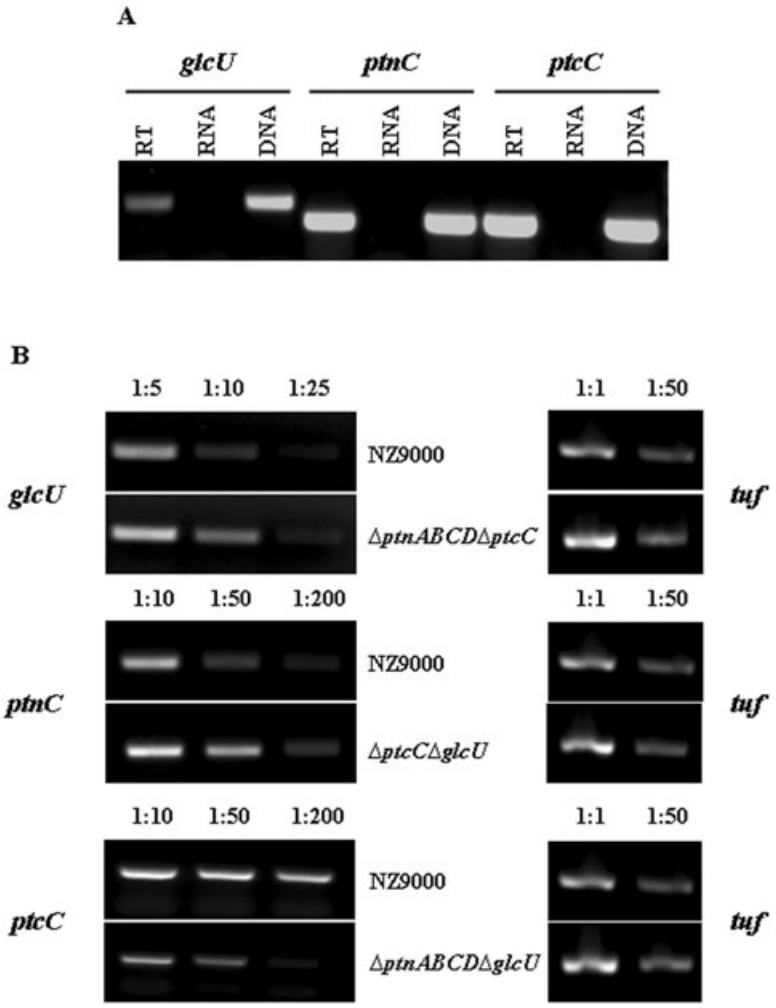

Fig. 3. Transcriptional studies of glucose transport systems in *L. lactis*. NZ9000 and derivatives were grown in CDM with 1% glucose (w/v) and without pH control (initial pH 6.5).
A. Transcription of glucose uptake system genes in strain NZ9000. PCR experiments were performed with chromosomal DNA from NZ9000 (DNA; positive control), RNA treated without reverse transcriptase (RNA; negative control) and cDNA resulting from the reverse transcription reaction (RT).
B. Comparison of gene transcription in NZ9000 and each of its isogenic mutants with a single transport system. The cDNA samples were diluted 1:5, 1:10, 1:25, 1:50 and 1:200, and used as template in the PCR analysis. RT-PCR experiments were performed using primers designed to amplify intragenic regions of *glcU* (590 bp), *ptnC* (443 bp), *ptcC* (464 bp) and *tufA* (471 bp). The elongation factor TU is required for continued translation of mRNA, and in *L. lactis* MG1363 is encoded by *tufA*. This housekeeping gene was used as a control in the RT-PCR experiments.

glucose/mannose:H^+ symporter (Paulsen *et al.*, 1998). Additionally, we speculate that GlcU is the primary non-PTS glucose permease in the *Lactobacillales*. This assumption is based on a screen of all available genomes of this taxonomic order for homologues of the other characterized bacterial glucose secondary carriers, the *E. coli* GalP (McDonald *et al.*, 1997) and the *Streptomyces coelicolor* GlcP (van Wezel *et al.*, 2005), which retrieved exclusively proteins annotated as xylose:H^+ symporters (data not shown).

The reported K_m values for glucose uptake via secondary carriers vary up to three orders of magnitude (mM or

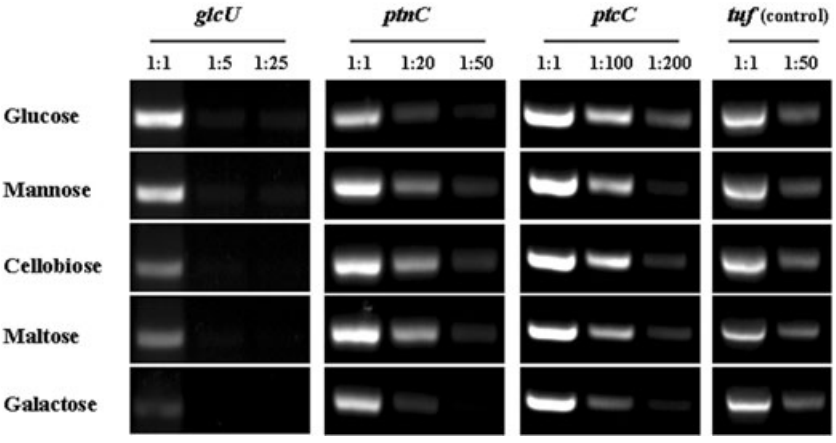

Fig. 4. Transcriptional studies of glucose transport systems in *L. lactis* NZ9000 grown in different sugars. Total RNA was extracted from cultures of *L. lactis* NZ9000 in CDM with 1% (w/v) glucose, mannose, cellobiose, maltose or galactose, and without pH control (initial pH 6.5). The cDNA samples were diluted 1:5, 1:20, 1:25, 1:50, 1:100 and 1:200, and used as template in the PCR analysis. RT-PCR experiments were performed using primers designed to amplify intragenic regions of *glcU* (590 bp), *ptnC* (443 bp), *ptcC* (464 bp) and the control gene *tufA* (471 bp).

μM), a broad range of affinity that is unrelated to the transporter family type (Cvitkovitch *et al*., 1995; Weisser *et al*., 1995; Wagner *et al*., 2000; Gosset, 2005; van Wezel *et al*., 2005; Parche *et al*., 2006). The lactococcal GlcU (K_m, 2.4 mM) clusters in the low-affinity group together with the non-PTS permease of *Bacillus megaterium* and the glucose facilitator (Glf) of *Zymomonas mobilis* (K_m values of 2.5–5 mM and 4.1 mM respectively). The low affinity of these systems for glucose was associated with a facilitated diffusion mechanism (Weisser *et al*., 1995; Wagner *et al*., 2000), but this explanation does not suit the data on lactococcal GlcU since we showed that transport via GlcU is dependent on the proton-motive force. Thus, our data are in line with the hypothesis that members of the glucose/ribose porter family operate by H^+ symport (Jack *et al*., 2001).

Internalization of glucose in *L. lactis* is either coupled to PEP-dependent phosphorylation (PTS^{Man} and PTS^{Cel}) or driven by the PMF (GlcU). Our data show that the lactococcal PTS^{Man} is a high-affinity, high-capacity system able to import both anomers of glucose, while PTS^{Cel} and GlcU are low-affinity transporters with a strong preference for β-glucose. Noteworthy is the clear dependency of glucose metabolism on the pathway used for taking up this sugar. As described before for wild-type strains (Neves *et al*., 2002a; 2006), consumption of glucose led to an increase in the FBP pool, of which the build-up rate and maximal concentration correlate with the glucose consumption rate. As opposed to the other *L. lactis* strains characterized here, the utilization of glucose, and the subsequent accumulation of FBP, in strain NZ9000Δ*ptnABCD*Δ*ptcC* (missing glucose PTSs) did not start immediately after glucose addition. This emphasizes the advantage conferred by PTSs for the rapid uptake of sugar after a starvation period; moreover, the high energetic efficiency of this uptake system is well documented (Postma *et al*., 1993). Loss of a PEP-consuming activity (either of the two glucose PTSs) resulted in a PEP potential (pools of 3-PGA and PEP) that is three- to fourfold higher than that in wild-type strains (Neves *et al*., 2002a; 2006). Curiously, the PEP potential in the strain devoid of glucose PTSs was lower than that in the isogenic strains with a single PTS (NZ9000Δ*ptcC*Δ*glcU* and NZ9000Δ*ptnABCD*Δ*glcU*). This feature is most likely associated with the low rate of FBP disappearance, which is indicative of a constriction at the level of glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase in the double-PTS mutant.

RT-PCR experiments using RNA from the parent strain *L. lactis* NZ9000 showed that the *glcU*, *ptnC* and *ptcC* genes are all transcribed under the conditions employed, suggesting that the three transport systems are present in this strain (Fig. 3A). Analysis of the consumption of α- and β-glucose in strain NZ9000 allowed us to assess the relative contributions of the high-affinity transporter PTS^{Man} and the low-affinity systems <PTS^{Cel}+GlcU>. The similar capacities found for the high- and the lumped low-affinity systems in strain NZ9000 indicate that their contributions to the total glucose uptake are comparable. The individual contributions of the two low-affinity transporters are difficult to evaluate. Assuming that the transcript level is proportional to the uptake activity, the higher level of *ptcC* transcript would imply that the PTS^{Cel} uptake rate in strain NZ9000 is higher than that found for the transporter in the absence of both other transporters (in strain NZ9000Δ*ptnABCD*Δ*glcU*) (Table 2). Along the same line of reasoning, strain NZ9000 would have a lower GlcU activity. Thus, it is possible that PTS^{Cel} contributes to a greater extent than GlcU to glucose uptake in NZ9000.

We showed that NZ9000 possesses two distinct PTSs and the secondary carrier GlcU for glucose uptake. The presence of two systems displaying similar kinetic properties, the PTS^{Cel} and the secondary carrier GlcU, is an intriguing feature. It is reasonable to question whether the systems are simultaneously operative or if the environmental conditions dictate the activity of each transporter. Indeed, it has been described that an acidic environment has a negative effect on PTS activity (Vadeboncoeur *et al*., 1991). Furthermore, it was shown that non-PTS permease(s) in *Streptococcus mutans* become functional at low pH (Hamilton and Ellwood, 1978; Cvitkovitch *et al*., 1995). Given that the fermentation of glucose to lactic acid progressively acidifies the lactococcal growth medium, we speculate that the presence of GlcU in *L. lactis* confers a competitive advantage to thrive at acidic pHs. It is anticipated that the insight into glucose transport derived from this work will assist in the design of *L. lactis* strains with improved traits for industrial applications.

Experimental procedures

Microbial strains and growth conditions

Strains and plasmids used in this work are listed in Table 3. Routinely, *L. lactis* strains were cultivated as batch cultures without aeration in M17 medium (Difco™, Sparks, MD, USA) supplemented with glucose (1% w/v) at 30°C. Deletion mutants were constructed in galactose-M17 (0.5% w/v), and the temperature was raised to 37°C for plasmid integration/excision. NMR studies were performed with cells grown in chemically defined medium (CDM) supplemented with 1% glucose (w/v), under anaerobic conditions in a 2 l fermenter (B. Braun Biostat® MD, B. Braun Biotech International, Melsungen, Germany). The CDM was gassed with argon for 60 min prior to inoculation (4–5% inoculum from a culture grown overnight), the pH was kept at 6.5 by automated addition of 10 M NaOH, and an agitation rate of 70 r.p.m. was used. RT-PCR studies were performed with cells grown in rubber-stoppered bottles (200 ml) in CDM without pH control (initial pH 6.5). The following sugars (1% w/v) were tested as carbon sources: glucose, mannose, cellobiose, maltose, galactose, xylose, rhamnose and ribose. Growth was not

Table 3. *L. lactis* strains and plasmids used in this study.

Strain/plasmid	Description	Reference
Strains		
LL302	*RepA*$^+$ MG1363, carrying a single copy of pWV01 *repA* in *pepX*	Leenhouts *et al.* (1998)
NZ9000	MG1363 derivative carrying *pepN*::*nisRK*	Kuipers *et al.* (1998)
NZ9000Δ*ptnABCD*	Derivative of NZ9000 containing a 1736 bp deletion in *ptnABCD*	Pool *et al.* (2006)
NZ9000Δ*ptcC*	Derivative of NZ9000 containing a 1254 bp deletion in *ptcC*	This work
NZ9000Δ*ptnABCD*Δ*ptcC*	Derivative of NZ9000Δ*ptnABCD* containing a 1254 bp deletion in *ptcC*	This work
NZ9000Δ*ptnABCD*Δ*ptcC*Δ*ytgBAH*	Derivative of NZ9000Δ*ptnABCD*Δ*ptcC* containing a 1716 bp deletion in *ytgBAH*	This work
NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*	Derivative of NZ9000Δ*ptnABCD*Δ*ptcC* containing a 864 bp deletion in *glcU*	This work
NZ9000Δ*ptnABCD*Δ*glcU*	Derivative of NZ9000Δ*ptnABCD* containing a 864 bp deletion in *glcU*	This work
NZ9000Δ*ptcC*Δ*glcU*	Derivative of NZ9000Δ*ptcC* containing a 864 bp deletion in *glcU*	This work
NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*(*glcU*$^+$)	Derivative of NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU* carrying pNZ8048-*glcU*	This work
Plasmids		
pORI280	Em^r, *lacZ*$^+$, *ori*$^+$ of pWV01, replicates only in strains providing *repA in trans*	Leenhouts *et al.* (1996)
pVE6007	Cm^r, temperature-sensitive derivative of pWV01	Maguin *et al.* (1992)
pNZ8048	Cm^r, nisin-inducible P*nisA*	de Ruyter *et al.* (1996)
pNZ8048-*glcU*	Cm^r, pNG8048 with *glcU* cloned in the NcoI/HindIII site	This work
pORI280-*ptcC′*	Em^r, derivative of pORI280 specific for integration in *L. lactis ptcC*	This work
pORI280-*ytgBAH′*	Em^r, derivative of pORI280 specific for integration in *L. lactis ytgBAH*	This work
pORI280-*glcU′*	Em^r, derivative of pORI280 specific for integration in *L. lactis glcU*	This work

Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

observed when xylose, rhamnose and ribose were used as sole carbon sources in CDM. When necessary, erythromycin or chloramphenicol was used at a final concentration of 5 μg ml^{-1}. For complementation, nisin (1 ng ml^{-1}) was used. Growth was monitored by measuring the optical density at 600 nm.

DNA techniques

General molecular techniques were performed essentially as described elsewhere (Sambrook *et al.*, 1989). Chromosomal and plasmid DNA were isolated by the method of Johansen and Kibenich (1992) and Birnboim and Doly (1979) respectively. *L. lactis* was transformed with plasmid DNA by electroporation as described by Holo and Nes (1995). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA) and Pwo polymerase and Taq polymerase were obtained from Roche Applied Science (Mannheim, Germany) and were used according to the supplier's instructions. PCR reactions were performed in a MyCyclerTM thermal cycler (Bio-Rad, Hercules, CA, USA). Primers (listed in Table S1) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Construction of L. lactis *mutant strains and plasmids*

Gene deletions were all performed in *L. lactis* NZ9000 and were constructed using a two-step homologous recombination method (Leenhouts *et al.*, 1996). This method does not leave antibiotic resistance markers in the chromosome, and multiple deletions in one strain can be accomplished. Chromosomal DNA of *L. lactis* NZ9000 was used as a template in PCR amplifications. The PTS^{Man} route was inactivated by deletion of *ptnABCD*-encoding enzyme II^{Man} as previously described (Pool *et al.*, 2006). To disrupt the PTS^{Cel}, a strain carrying a deletion in *ptcC*, the membrane-bound domain IIC of enzyme II^{Cel}, was constructed as follows: a 1253 bp deletion in *ptcC* (1338 bp) was made using the primer pairs ptcC1/ptcC2 and ptcC3/ptcC4. Genes *ytgBAH* (locus tags llmg_2146, llmg_2145 and llmg_2143 in *L. lactis* MG1363 genome sequence) (Wegmann *et al.*, 2007) were inactivated by deletion in strain NZ9000Δ*ptnABCD*Δ*ptcC* using the primers ytgBAH1/ytgBAH2 and ytgBAH3/ytgBAH4. *L. lactis* NZ9000Δ*glcU*, carrying only the last 23 bp of llmg_2561 (*glcU*), was engineered using the primer pairs glcU1/glcU2 and glcU3/glcU4. The deletions were confirmed by PCR and Southern blotting analysis (Fig. S2). For a complementation study, the *glcU* gene (888 bp) was amplified by PCR using *L. lactis* NZ9000 DNA as template and primer pairs GlcU-fw and GlcU-rev (Table S1). The NcoI/HindIII-restricted PCR product was cloned in NcoI/HindIII-restricted pNZ8048. The resulting plasmid pNZ8048[*glcU*] was transformed into strain NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*, yielding strain NZ9000Δ*ptnABCD*Δ*ptcC*Δ*glcU*(*glcU*$^+$).

[^{14}C]-glucose transport studies

All strains were grown in M17 medium supplemented with 1% glucose, without pH control. Cells were harvested at the mid-exponential phase of growth, washed twice in KP$_i$ buffer (5 mM, pH 6.5) and suspended in the KP$_i$ buffer (50 mM, pH 6.5). Initial glucose uptake rates were measured at 30°C in 100 μl of cell suspensions with appropriate optical densities at 600 nm. [U-^{14}C] glucose was added to a final concentration of 0.001–20 mM (specific activity 0.02–19 μCi μmol^{-1}). Uptake assays were performed essentially as described by Wolken *et al.* (2006), except that KP$_i$ buffer (50 mM, pH 6.5) was used to wash the filters. For kinetic analysis of glucose transport via GlcU (in NZ9000Δ*ptnABCD*Δ*ptcC*), the cell sus-

pension was incubated with 2 mM arginine prior to the addition of labelled glucose. The kinetic parameters (K_m and V_{max}) for glucose uptake were estimated by fitting the data using non-linear least squares regression analysis (Excel solver, Microsoft Excel 2003) to the Michaelis–Menten equation. Glucose uptake was also evaluated in the presence of 20 μM TCS and 150 μM CCCP. The effect of protonophores was studied as follows: cell suspensions of NZ9000Δ*ptnABCD*Δ*ptcC* were incubated with the compound for 3 min at 30°C. [^{14}C]-glucose was added to a final concentration of 150 μM and the uptake was stopped after 60 s. The influence of ethanol (2%, v/v), the solvent of CCCP and TCS, was also examined. Transport competition experiments were performed with energized NZ9000Δ*ptnABCD*Δ*ptcC* (in the presence of 2 mM arginine). Glucose uptake was measured in cell suspensions with 0.5 mM glucose and 100-fold excess of the following carbohydrates: ribose, rhamnose, mannose, xylose, arabinose, cellobiose and galactose. The competition assays were performed in triplicate using cells from two independent cultures.

In vivo *NMR experiments*

Cells were grown in CDM containing 1% glucose (w/v) and suspensions were prepared and made anaerobic as described elsewhere (Neves *et al*., 1999). *In vivo* NMR experiments were performed using an online system and glucose specifically labelled with ^{13}C on carbon one (20 mM) as substrate (Neves *et al*., 1999; 2002a). In strain NZ9000Δ*ptnABCD*Δ*ptcC*, [1-^{13}C]-glucose utilization was also studied following energization of the resting cells with arginine (2 mM). *In vivo* ^{13}C-NMR spectra were acquired at 125.77 MHz using a quadruple nuclei probe head at 30°C on a Bruker AVANCE II 500 MHz spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) as described before (Neves *et al*., 1999). Lactate was quantified in the NMR-sample extract by ^{1}H-NMR in a Bruker AMX300 (Bruker BioSpin GmbH). The concentration of other metabolites was determined in fully relaxed ^{13}C spectra of the NMR-sample extracts as previously described (Neves *et al*., 2002b). Each experiment was repeated at least twice and the results were highly reproducible.

Kinetics of α- and β-glucose consumption

A mathematical model for the consumption of α- and β-glucose was developed using Michaelis–Menten formalism, and taking into account the first-order kinetics of anomerization. The model considers that each anomer competes with the other for the free transporter (T_{free}), leading to the formation (k_{+1}) of a complex ([αGlc-T] or [βGlc-T]). Binding to the transporter is reversible and dissociation can occur without transport (k_{-1}), or the glucose anomer is transported and released to the intracellular space (k_2) (schematic representation in Fig. S3). The uptake rate of the individual anomers is given by

$$v^{\alpha}=\frac{V_{max}\cdot[\alpha Glc]}{K_{af}^{\alpha}\cdot\left(1+\frac{[\beta Glc]}{K_{af}^{\beta}}\right)+[\alpha Glc]} \qquad v^{\beta}=\frac{V_{max}\cdot[\beta Glc]}{K_{af}^{\beta}\cdot\left(1+\frac{[\alpha Glc]}{K_{af}^{\alpha}}\right)+[\beta Glc]}, \tag{1}$$

where $K_{af}^{n}=\frac{k_{-1}^{n}+k_{2}^{n}}{k_{+1}^{n}}$, $V_{max}=k_2\cdot T$ for $n=\alpha$ or β. Moreover, it was assumed that the V_{max} values for α- and β-glucose were identical, i.e. $k_2^{\alpha}=k_2^{\beta}$. Therefore, V_{max} represents the maximum capacity of glucose consumption, regardless the anomeric form that is taken up. The parameters K_{af}^{α} and K_{af}^{β} are the affinity constants for utilization of each glucose anomer. It is implicitly assumed that transport is the rate limiting step in the metabolism of glucose and the actual kinetics of glucose uptake may be affected by subsequent metabolism. Consumption of α- and β-glucose by *L. lactis* as monitored by ^{13}C-NMR was in some cases slower than the anomerization rate, thus requiring the consideration of the anomerization step. The first-order rate constants of glucose anomerization were determined by acquiring sequences of ^{13}C-NMR spectra of 20 mM α-[1-^{13}C]-glucose dissolved in the same buffer used for *in vivo* NMR experiments (50 mM KP_i, pH 6.5), at 30°C. Under these conditions the equilibrium molar percentages of α-glucose and β-glucose were 38.2% and 62.8% respectively. The first-order rate constants were 0.108 ± 0.001 min^{-1} for the conversion of α into β and 0.063 ± 0.001 min^{-1} for the conversion of β into α.

The profile of glucose consumption by *L. lactis* was approximately sigmoidal, i.e. at the initial stage, immediately after glucose addition, glucose uptake proceeded at a rate lower than maximal and this feature was especially apparent in the strain transporting glucose via the GlcU permease only (see above). To account for this behaviour the V_{max} of glucose transport was multiplied by the following function:

$$\varphi=\begin{cases}a+b\cdot t+c\cdot t^2, & a+b\cdot t+c\cdot t^2\le 1\\ 1, & a+b\cdot t+c\cdot t^2>1\end{cases}. \tag{2}$$

The parameters *a*, *b* and *c* were determined so that $0\le\varphi\le 1$, and φ increases from *a* to 1 monotonically with time, *t*. The model was implemented in MATLAB v7.3.0 (The MathWorks, Natick, MA, USA) as a set of ordinary differential equations.

$$\begin{cases}\frac{\partial[\alpha Glc]}{\partial t}=k_{\beta\to\alpha}\cdot[\beta Glc]-k_{\alpha\to\beta}\cdot[\alpha Glc]-\varphi\cdot\frac{V_{max}\cdot[\alpha Glc]}{K_{af}^{\alpha}\cdot\left(1+\frac{[\beta Glc]}{K_{af}^{\beta}}\right)+[\alpha Glc]}\\ \frac{\partial[\beta Glc]}{\partial t}=k_{\alpha\to\beta}\cdot[\alpha Glc]-k_{\beta\to\alpha}\cdot[\beta Glc]-\varphi\cdot\frac{V_{max}\cdot[\beta Glc]}{K_{af}^{\beta}\cdot\left(1+\frac{[\alpha Glc]}{K_{af}^{\alpha}}\right)+[\beta Glc]}\end{cases}. \tag{3}$$

The parameters (V_{max}, K_{af}^{α}, K_{af}^{β}, *a*, *b* and *c*) were determined by fitting the model to the α- and β-glucose consumption profiles determined experimentally. A simulated annealing algorithm was used to ensure that the minimum found was indeed a global minimum. The differential equations were solved using the *ode23s* function and the non-linear regression performed using the *lsqnonlin* function.

RT-PCR experiments

Lactococcus lactis NZ9000 and derivatives were grown as described above. Total RNA was isolated from cells at the mid-exponential phase of growth using the SV total RNA isolation system (Promega, Woods Hollow Road, MA, USA), with the following modifications: incubation with lysozyme (5 mg ml^{-1}, 20 min, 37°C) preceded the first step of the kit protocol, and an additional incubation step with the kit DNase I (1.5 h, 24°C) was required to remove chromosomal DNA. Total RNA (1 μg), dNTPs (final concentration of 0.5 mM) and random oligonucleotides (12 μg ml^{-1}) (Invitrogen, Carlsbad, CA, USA) were heated to 65°C for 5 min and chilled on ice. Dithiothreitol (final concentration 5 μM), first-strand RT buffer and Superscript III (1/20; v/v) (Invitrogen, Carlsbad, CA, USA) were added and samples were incubated for 5 min at 25°C, 60 min at 50°C and 15 min at 70°C for enzyme inactivation. A parallel sample was treated in the same way, except for the addition of enzyme. cDNA was subsequently used 1/30 (v/v) in standard PCR reactions. To test for contamination of RNA with DNA, the RNA samples without reverse transcriptase were used as negative controls for all conditions tested. Chromosomal *L. lactis* NZ9000 DNA was used as positive control for the PCR reaction. The primer pairs (Table S1) were designed to amplify internal fragments of *glcU* (glcU_RT1/glcU_RT2), *ptnC* (ptnC_RT1/ptnC_RT2), *ptcC* (ptcC_RT1/ptcC_RT2) and *tufA* (tuf_RT1/tuf_RT2). *L. lactis tufA*, a housekeeping gene coding for the elongation factor TU required for continued translation of mRNA, was used as control. RT-PCR was performed twice with RNA isolated from independent cultures.

Transcriptome analysis

The levels of mRNA in NZ9000 and a derivative strain lacking PTS^{Man} and PTS^{Cel} glucose transporters were compared by transcriptome analysis using full-genome amplicon-based *L. lactis* IL1403 DNA microarrays (Kuipers *et al.*, 2002). The experiments were performed essentially as described by van Hijum *et al.* (2005), with the modifications introduced by Pool *et al.* (2006).

Chemicals

[1-^{13}C]-glucose (99% enrichment) and [U-^{14}C]-glucose (287 mCi $mmol^{-1}$) were obtained from Campro Scientific (Veenendaal, the Netherlands) and Amersham Biosciences (GE Healthcare, Europe) respectively. All other chemicals were reagent grade.

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