João Manuel Pereira Jorge

Degree in Biotechnology

Bioengineering of *Lactococcus lactis* through modulation of its major glucose transporter

Dissertation to obtain the Master Degree in Biotechnology

Supervisor: Ana Rute Neves, Ph.D., ITQB-UNL
Co-Supervisor: Paula Gaspar, Ph.D., ITQB-UNL

Thesis evaluation committee:

Chairman: Prof. Dr. Pedro Miguel Ribeiro Viana Baptista
Main Opponent: Prof. Dr. Isabel Maria Godinho de Sá Nogueira
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Abstract

Glucose is abundant in nature, reasonably cheap, and has been the substrate of choice for biotechnological applications in *Lactococcus lactis*. This bacterium possesses two phospho*enol*pyruvate:phosphotransferase systems (PTS), the mannose-PTS (PTS^{Man}) and the cellobiose-PTS (PTS^{Cel}), and a secondary carrier (GlcU) for glucose uptake. From the three, PTS^{Man} is considered the main glucose transporter. To assess the contribution of each transporter to the overall glucose metabolism, three single deletion mutants [Δ*ptnABCD* (PTS^{Man-}), Δ*ptcBA* (PTS^{Cel-}) and Δ*glk* ($GlcU^{-}$)] were constructed and characterized. Two types of colonies were isolated during inactivation of *glk* and *ptcBA*. Isolates 1 displayed only a modest effect on glucose transport and metabolism, while the isolates 2 were drastically affected. Sequence analysis of the PTS^{Man} encoding operon, revealed single amino acid changes in $EIIC^{Man}$ of Δ*glk* (isolate 2) and $EIID^{Man}$ of Δ*ptcBA* (isolate 2).

To ascertain that the random mutations conferred the observed phenotype, the identified single base changes were introduced in *L. lactis* NZ9000 (wild-type) and isolates 1 of Δ*glk* or Δ*ptcBA* by directed mutagenesis. NZ9000 derivates displayed low growth and glucose consumption rates, and a preference for β-glucose. These effects were accentuated when in combination with *glk* or *ptcBA* inactivation. In general, $EIIC^{Man}$ mutation had a bigger impact on glucose metabolism as compared to that of $EIID^{Man}$. Although its affinity for glucose was maintained, PTS^{Man} capacity was severely affected by the mutations.

One of the main metabolic consequences of the point-mutations was an increased pyruvate, and eventually a phospho*enol*pyruvate (PEP), pool; a feature that was exploited for the production of succinate. However, the attempts made to overexpress PEP and pyruvate carboxylases from *Corynebacterium glutamicum* in *L. lactis* had a limited success.

In conclusion, this work disclosed key amino acid residues in the functionality of PTS^{Man} in *L. lactis*.

Keywords: *Lactococcus lactis*, glucose transporters, glucose metabolism, succinate.

Resumo

A glucose é um açúcar abundante na natureza, razoavelmente barato e é o substrato preferido em processos biotecnológicos que usam *Lactococcus lactis*. Esta bactéria possui dois sistemas tipo fosfo*enol*piruvato:fosfotransferase (PTS), o PTS-manose (PTS^{Man}) e o PTS-celobiose (PTS^{Cel}), e um transportador secundário (GlcU) para transportar de glucose. Dos três, o PTS^{Man} é considerado o transportador principal. Para avaliar a contribuição de cada transportador no metabolismo global de glucose, foram construídos e caracterizados três mutantes [Δ*ptnABCD* (PTS^{Man-}), Δ*ptcBA* (PTS^{Cel-}) e Δ*glk* ($GlcU^{-}$)]. Durante a inativação dos genes *glk* e *ptcBA*, isolaram-se dois tipos de colónias. Os isolados 1 mostraram apenas um efeito modesto no transporte e metabolismo da glucose, enquanto que os isolados 2 estavam drasticamente afetados. A sequenciação do operão que codifica o PTS^{Man}, revelou a troca de um aminoácido nas subunidades $EIIC^{Man}$ da Δ*glk* (isolado 2) e $EIID^{Man}$ da Δ*ptcBA* (isolado 2).

Para verificar se as mutações aleatórias conferiam o fenótipo observado, as substituições identificadas foram introduzidas em *L. lactis* NZ9000 (estirpe selvagem) e nos isolados 1 da Δ*glk* ou Δ*ptcBA* através de mutagénese dirigida. Os mutantes NZ9000 exibiram taxas de crescimento e de consumo de glucose menores, e uma preferência por β-glucose. Estes efeitos acentuaram-se em combinação com a inativação da *glk* ou *ptcBA*. Em geral, a mutação na $EIIC^{Man}$ teve um impacto maior no metabolismo de glucose que a mutação na $EIID^{Man}$. Apesar de ter mantido a sua afinidade por glucose, a capacidade do PTS^{Man} foi severamente afetada pelas mutações.

Uma das principais consequências metabólicas das mutações pontuais foi o aumento dos níveis de piruvato e eventualmente fosfo*enol*piruvato (PEP); uma característica que foi explorada para a produção de succinato. Contudo, as tentativas feitas para super-expressar os genes que codificam a PEP e a piruvato carboxilases de *Corynebacterium glutamicum*, não foram bem sucedidas.

Em conclusão, este trabalho permitiu identificar aminoácidos chave na funcionalidade do PTS^{Man} de *L. lactis*.

Palavras-chave: *Lactococcus lactis*, transportadores de glucose, metabolismo de glucose, sucinato.

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List of abbreviations, acronyms and symbols

Abbreviation	Full Form
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CDM	Chemically defined medium
D_2O	Deuterium oxide also known as deuterated water
DHAP	Dihydroxyacetone phosphate
DTT	Dithiothreitol
EMP	Embden-Meyerhof-Parnas
EI	Enzyme I
EII	Enzyme II
FBP	Fructose 1,6-bisphosphate
GAP	Glyceraldehyde 3-phosphate
GLK	Glucokinase
GRAS	Generally regarded as safe
G6P	Glucose 6-phosphate
HPLC	High Performance Liquid Chromatography
HPr	Histidine-containing phosphocarrier protein
K_m	Michaelis-Menten affinity constant
KP_i	Potassium phosphate
LAB	Lactic acid bacteria
LB	Luria-Bertani medium
LDH	Lactate dehydrogenase
Ln	Natural logarithm
MAMA	Mismatch amplification mutation assay
MDH	Malate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide
NADH	Dihydronicotinamide adenine dinucleotide

NBDs	Nucleotide-binding domains
ND	Not detected
NICE system	Nisin inducible controlled expression system
NMR	Nuclear magnetic resonance
OAA	Oxaloacetate
OD	Optical density
PCR	Polymerase chain reaction
PEP	Phospho*enol*pyruvate
3-PGA	3-Phosphoglycerate
P_i	Inorganic phosphate
PTS	Phospho*enol*pryruvate:phosphotransferase system
PTS^{Cel}	Cellobiose-PTS
PTS^{Man}	Mannose-PTS
PYC	Pyruvate carboxylase
PPC	Phospho*enol*pyruvate carboxylase
SBP	Sugar binding proteins
SC	Secondary carriers
SD	Standard deviation
TCA	Tricarboxylic acid
t/n	Generation time
TMD	Transmembrane domain
μ	Specific growth rate
V_{max}	Maximum velocity

1. Introduction

1. 1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) emerged as group in the beginning of the 20th century, being one of the most industrially important groups of bacteria nowadays (Pfeiler and Klaenhammer, 2007). The LAB group is constituted by bacteria that are closely related physiological, morphological, metabolic and also phylogenetically, and which was first defined by Orla-Jensen in 1919. Since then these bacteria have been unequivocally accepted as: Gram-positive, non-sporing microaerophilic bacteria whose the major fermentation product from carbohydrates is lactate (Kandler, 1983). Some recent revisions have proposed the inclusion of new members and the LAB group is now composed by the following genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Classification of LAB genera is based on the following characteristics: mode of glucose fermentation, configuration of lactic acid produced, acid or alkaline tolerance, ability to grow at high salt concentrations, morphology and growth at certain temperatures. For some of newly described genera, like *Carnobacterium*, characteristics as fatty acid composition and motility are used in the classification. *Carnobacteria*, *Lactobacilli* and some *Weissella* are rods while the remaining genera are cocci (Khalid, 2011; Liu *et al.*, 2009).

LAB can only obtain ATP by fermentation (normally of sugars) because they lack the capacity to synthesize cytochromes and porphyrins (components of respiratory chains). As fermentative organisms LAB grows happily under anaerobic conditions, but they can also tolerate oxygen. There are two main sugar fermentation pathways in LAB, the homofermentative and the heterofermentative. Homolactic genera as *Lactococcus* or *Streptococcus* use the Embden-Meyerhof-Parnas (EMP) pathway to convert glucose to pyruvate that is further reduced to lactate. Genera like *Leuconostoc* or *Oenococcus* are heterofermentative LAB, and metabolize glucose via the 6-phosphogluconate/phosphoketolase pathway producing lactate, acetate and ethanol (Fig. 1.1). The end-product profiles of some lactic acid bacteria can be significantly altered by different growth conditions, and these changes are attributed to difference in pyruvate metabolism and/or the use of external electron acceptors such as oxygen and organic compounds (Khalid, 2011; Schleifer *et al.*, 1995).

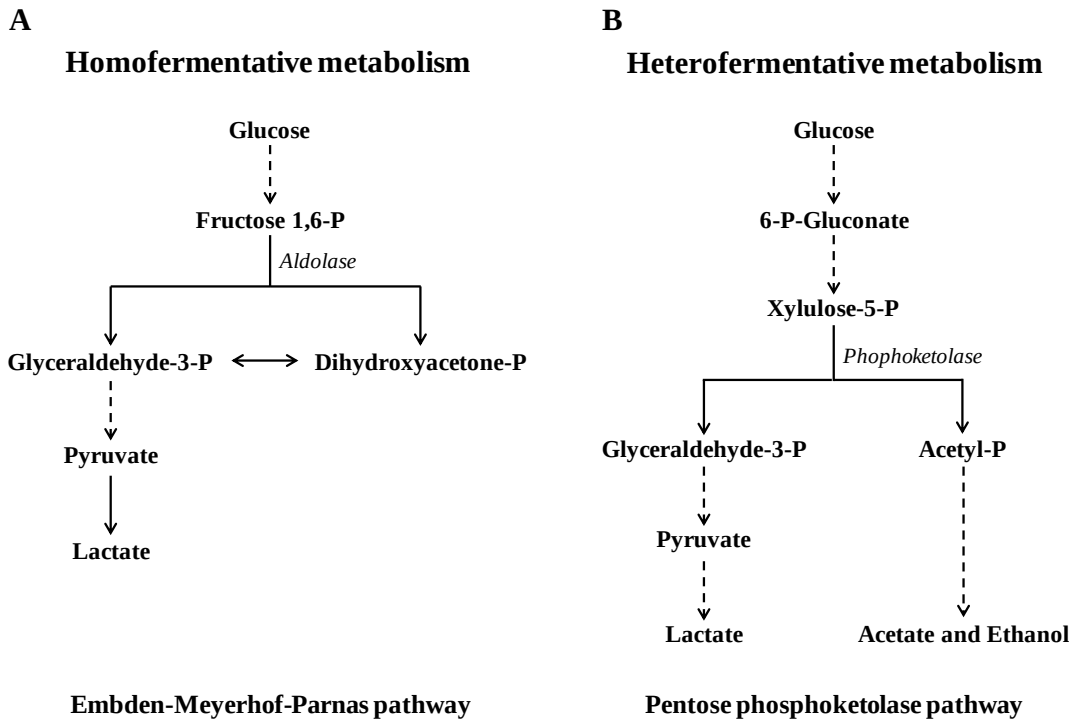

Fig. 1.1 – Schematic representation of the main pathways of glucose fermentation in lactic acid bacteria. Adapted from (Kandler, 1983).

LAB can survive in distinct biotopes, like foods and plants or even the human body (Levering *et al.*, 2012). The impact of LAB on our daily life is significant. This group of organisms includes important pathogens, like some *Streptococcus* species, and numerous non-pathogenic species, like *Lactococcus lactis*, which are very important in the manufacturing of fermented milk products, meat and vegetables or even wine. The fast growth of LAB from the available carbon source, implies the acidification of the food, and therefore, providing efficient and natural means of food preservation (Kleerebezem and Hugenholtz, 2003; Konings *et al.*, 2000). Moreover, many LAB are able to secrete antimicrobial peptides which are used to extend the shelf-life of the fermented products (de Vos *et al.*, 1995). In addition to preservation, LAB also contribute to other product characteristics, such as flavour (e.g. acetaldehyde), texture (e.g. exopolysaccharides) and nutritional value. Their potential as probiotics (e.g. milk drinks) or for the production of protein ingredients (e.g. nisin) and therapeutic proteins (e.g. interleukin-10) has also been demonstrated (de Vos and Hugenholtz, 2004; Kleerebezem and Hugenholtz, 2003; Papagianni, 2012).

1. 2 The model organism *Lactococcus lactis*

Lactococcus lactis is a non-pathogenic low-GC Gram-positive bacterium closely related to the genus *Streptococcus* and the best-characterized member of LAB (Bolotin *et al.*, 2001). This

bacterium is widely used in starter cultures for the manufacture of fermented dairy products, such as cheese and buttermilk. Generally regarded as safe (GRAS), *L. lactis* is a facultative anaerobe that lacks cytochromes of the respiratory chain, non-motile, catalase-negative, non-sporulating cocci (cells are spherical or ovoid), and whose main fermentation product is lactate. It forms the genus *Lactococcus* together with the species *L. garvieae*, *L. raffinolactis*, *L. piscium* and *L. plantarum*. The *L. lactis* species can be divided in three subspecies: *L. lactis* subs. *cremoris*, *L. lactis* subs. *lactis* and *L. lactis* subs. *hordniae* (Schleifer, 1985; Williams *et al.*, 1990).

The simple carbon and energy metabolism, the small genome, the genetic accessibility and the fast growing properties, makes *L. lactis* an excellent model organism to study metabolism, physiology and molecular biology of LAB (de Vos and Hugenholtz, 2004). In the past years many genetic tools including constitutive and regulated gene expression systems, gene replacement and transformation protocols have been developed for *L. lactis* (D'Souza *et al.*, 2012; Kleerebezem and Hugenholtz, 2003; Kuipers *et al.*, 1998; Leenhouts *et al.*, 1996; Pinto *et al.*, 2011a; Pinto *et al.*, 2011b; Solem and Jensen, 2002). Moreover, the genome sequences of *L. lactis* subs. *lactis* CV56, IL1403, KF147 and the subs. *cremoris* MG1363, NZ9000, A76 and SK11 are already determined (Bolotin *et al.*, 2012; Bolotin *et al.*, 2001; Gao *et al.*, 2011; Linares *et al.*, 2010; Makarova *et al.*, 2006; Siezen *et al.*, 2010; Wegmann *et al.*, 2007). Altogheter, the availability of these tools and information led to the use of lactococci beyond food fermentations, opening a way for new applications of *L. lactis*.

Different metabolic engineering approaches for the production of high-value compounds which can improve the flavour or health benefit of fermented products by *L. lactis* have been reported (Table 1.1) (de Vos and Hugenholtz, 2004; Gaspar et al., 2008; Kleerebezem and Hugenholtz, 2003). The manipulation of *L. lactis* has focused primarly in pyruvate metabolism re-routing to produce important fermentation end-products as diacetyl (buttermilk flavour), acetaldehyde (yoghurt flavour), alanine (natural sweetner), mannitol (low-calorie sweetner) or 2,3-butanediol (chemical feedstock) (Gaspar *et al.*, 2011; Gaspar *et al.*, 2004; Hols *et al.*, 1999; Hugenholtz *et al.*, 2000; Liu *et al.*, 2005). The efficient production of these compounds frequently requires the inactivation of the lactate dehydrogenase (LDH), a key enzyme in lactococcal metabolism which converts pyruvate to lactate. Additionaly, metabolic enginnering of complex biosynthetic pathways leading to the production of exopolysaccharides and vitamins (riboflavin and folate) were also accomplished (Boels *et al.*, 2003; Burgess *et al.*, 2004; Sybesma *et al.*, 2003). Other engineering strategies have targeted hexose metabolism (e.g. glucose production from lactose or galactose removal), products requiring heterologous gene expression (e.g. trehalose, hyaluronic acid or strawberry flavour) or a wide number of proteins with clinical interest (Carvalho *et al.*, 2011; Chien and Lee, 2007; Hernandez *et al.*, 2007; Morello *et al.*, 2008; Neves *et al.*, 2010; Pool *et al.*, 2006; Termont *et al.*, 2006). As

demonstrated, *L. lactis* is undoubtedly a very important organism with diverse applications in industrial and clinical processes.

Table 1.1 – Examples of *L. lactis* biotechnological applications. Adapted from (de Vos and Hugenholtz, 2004).

Engineering strategy	Product	Application	Reference
Pyruvate metabolism	Alanine	Natural sweetner	(Hols *et al.*, 1999)
	Diacetyl	Flavour ingredient	(Hugenholtz *et al.*, 2000)
	Mannitol	Low-calorie sweetner	(Gaspar *et al.*, 2004)
	Acetaldehyde	Flavour ingredient	(Liu *et al.*, 2005)
	2,3-Butanediol	Chemical feedstock	(Gaspar *et al.*, 2011)
Hexose metabolism	Glucose (from lactose)	Useful for generating *in situ* sweetened products from milk-derived medium	(Pool *et al.*, 2006)
	Galactose removal	Dairy products for people with galactosemia	(Neves *et al.*, 2010)
Complex pathways	Exopolysaccharides	Development of texture in fermented products	(Boels *et al.*, 2003)
	Riboflavin	Nutraceutical	(Burgess *et al.*, 2004)
	Folate	Nutraceutical	(Sybesma *et al.*, 2003)
Other products (resulting from heterologous gene expression)	Trehalose	Celular protector against several stressful conditions	(Carvalho *et al.*, 2011; Termont *et al.*, 2006)
	Hyaluronic acid	Therapeutic applications	(Chien and Lee, 2007)
	Strawberry flavors	Flavour ingredient	(Hernandez *et al.*, 2007)

1. 3 Sugar transporters in bacteria

In bacteria, the transport includes the uptake of nutrients, as well as the excretion of metabolic end-products or toxic compounds, energy generation and pH homeostasis. The cell membrane is very impermeable to the majority of solutes, requiring the action of specific carrier proteins to

do translocation across this barrier. Transport of solutes can be driven by a favourable concentration gradient, a process known as passive-mediated transport or facilitated diffusion, or in most cases can occur against the concentration gradient, in a process that requires an energy source (Lolkema *et al.*, 1998). Depending of the energy source used there are three mechanisms of sugar transport in bacteria; primary transport systems, secondary carriers and group translocators (Fig. 1.2).

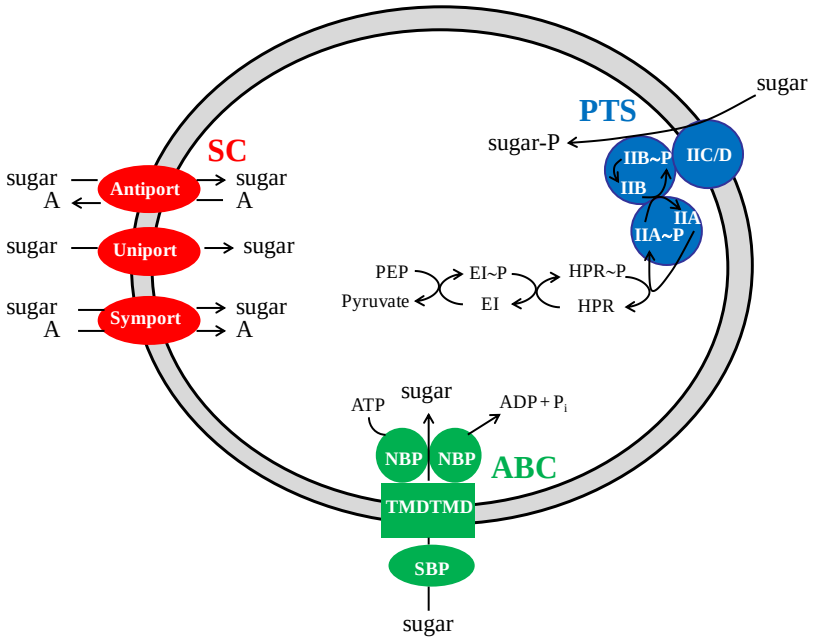

Fig. 1.2 - The three classes of sugar uptake systems in bacteria. The ATP-binding cassette importers (ABC transporters) are typically constituted by a sugar binding protein (SBP), two transmembrane domains/proteins (TMD) and two nucleotide binding domains/proteins (NBP). The secondary carriers (SC) include symporters, uniporters and antiporters. The phosphorylation cascade of the phospho*enol*pyruvate: carbohydrate phosphotransferase system (PTS) is shown, and includes the general PTS proteins, histidine-containing phosphocarrier protein (HPr) and enzyme I (EI) and the carbohydrate specific enzyme II (EII) complex (in this case IIABCD). Adapted from (Castro, 2009).

ATP-binding cassette transporters - In the primary transport system, the transport of the solutes across the membrane is done at the expense of adenosine triphosphate (ATP), which is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). In these systems light or chemical energy is converted into electrochemical energy (Poolman and Konings, 1993). The discovery of transport-related binding proteins in bacteria goes back to the end of 1960s, and it is clear now that the bacterial ATP-binding cassette (ABC) transporters and their homologues in eukaryotic cells constitute one of the largest superfamilies of proteins known. Usually, ABC proteins couple ATP hydrolysis to physical movements of small molecules or also proteins across the membrane. (Ehrmann *et al.*, 1998; Lee *et al.*, 2007). ABC transporters can function as importers, which bring nutrients and other molecules into cells, or as exporters,

driving toxins, drugs and lipids out of the cells. The importers seem to be present only in prokaryotic organisms whereas exporters are identified in eukaryotes and prokaryotes.

The architecture of ABC transporters consists of two hydrophobic transmembrane domains (TMDs) that are involved in the translocation across the membrane and two hydrophilic nucleotide-binding domains (NBDs) that interact at the cytoplasmic surface to provide the energy for active transport (Davidson and Chen, 2004; Rees *et al.*, 2009). In prokaryotes, subtrate translocation by ABC transporters is also dependent of another protein component, the sugar binding protein (SBP), that is responsible for the high-affinity uptake of solutes. In Gram-positive bacteria and Archaea, this protein is anchored to the cytoplasmic membrane and exposed on the cell surface, and in Gram-negative bacteria is located in the periplasm (Ehrmann *et al.*, 1998).

Sugar transport via ABC transporters is not the most frequent system of transport in LAB, nevertheless, there are some reports on the use of these systems to translocate carbohydrates. As an example, the disaccharide maltose can be transported by an ABC transporter in *L. lactis* (Law *et al.*, 1995).

Secondary transport systems - The secondary transport include symporters, uniporters and antiporters. In this type of transport the free energy for accumulation of a solute is supplied by electrochemical gradients of other solutes, including ions (Poolman and Konings, 1993).

In uniport systems, solute transport is independent of any coupling solute. In antiport systems there are coupled movements of solutes in opposite directions. Symporters mediate the coupled transport of two or more solutes in the same direction (Pool, 2008; Poolman and Konings, 1993). As the ABC transporters, the secondary transport systems are not common in LAB. The lactose transporter (LacS) of *S. thermophilus* is the best studied example of a secondary transport protein in LAB. LacS can import lactose either by symport or by antiport systems (Foucaud and Poolman, 1992).

Phospho*enol*pyruvate:carbohydrate phosphotransferase systems - The third mechanism of carbohydrate transport present in bacteria is the phospho*enol*pyruvate (PEP): carbohydrate phosphotransferase system (PTS), which was discovered in *E. coli* more than forty years ago (Kundig *et al.*, 1964). This system is involved in both transport and phosphorylation of a large number of carbohydrates and uses the energy provided by the glycolytic intermediate PEP to catalyze carbohydrate uptake (Postma *et al.*, 1993).

Comparing with non-PTS transporters, this system is more efficient in terms of energy requirements since it only uses one PEP molecule for the translocation and phosphorylation of the sugar. In energetic terms one molecule of PEP is equivalent to one ATP molecule, because, durying glycolysis, one ATP molecule is derived from one PEP molecule in the pyruvate kinase

reaction. In non-PTS systems, more than one ATP must be expended in transport and subsequent ATP-dependet phosphorylation of the incoming sugar. The energetic benefit of PTSs has been evoked as reason for these systems being mainly found in obligate and facultative anaerobic bacteria, that synthesize ATP only by substrate-level phosphorylation (Postma *et al.*, 1993).

The phosphoryl group transference from PEP to the incoming sugar occurs in a phosphorylation cascade involving several players (Fig. 1.3).

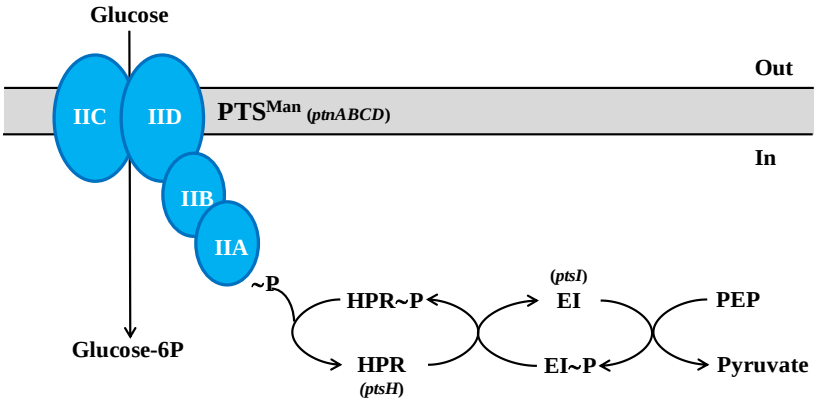

Fig. 1.3 - The phospho*enol*pyruvate-dependent:carbohydrate phosphotransferase system (PEP:PTS). In this example glucose is the imported molecule, the EII-conformation of a mannose-class EII is expected. The phosphate cascade from phospho*enol*pyruvate via enzyme I (EI, encoded by *ptsI*), histidine-containing phosphoscarier protein (HPr, encoded by *ptsH*) and enzyme IIA (EIIA, encoded by part of *ptnABCD* in *L. lactis* MG1363) to the imported sugar is shown. Adapted from (Pool, 2008).

The PTS system is a complex formed by the enzyme II (EII), a sugar-specific permease with three or four domains, and general cytoplasmic energy-coupling proteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr), which participate in the phosphorylation of all PTS sugars in a given organism (Table 1.2) (Barabote and Saier, 2005). The phosphoryl group of PEP is transferred to EI (encoded by *ptsI*), that subsequently transfers the phosphate to the histidine residue at position 15 of the HPr (encoded by *ptsH*), which further transfers the phosphate for EII, that will catalyze the phosphorilation of the imported carbohydrate. In the most common PTSs, the EII complex is a single membrane-bound protein composed of three domains (A, B and C). The EIIA and the EIIB form the hydrophilic domains. During the phosphate transfer, the EIIA domain is phosphorylated by HPr~P at the N-3 position of a histidyl residue that subsequently phosphorylate the EIIB domain at the N-1 position of a histidyl residue. The IIC is the membrane-bound hydrophobic domain, that makes sugar translocation across the membrane. The organization of the different domains depends on the PTS family. In the case of mannose-PTS (PTS^{Man}), the IIA and IIB domains form a single

soluble polypeptide, and there are two membrane-bound hydrophobic domains (IIC and IID) (Postma *et al.*, 1993).

Table 1.2 – PTS components. Adapted from (Barabote and Saier, 2005).

Protein or process	Description
IIC	Permease and receptor (sugar specific)
IIB	Direct phosphoryl donor (permease specific)
IIA	Indirect phosphoryl donor (family specific)
IID	Mannose family-specific auxiliary protein
EI	General energy-coupling protein. Phosphorylated at the N-3 position of a histidyl residue of EI during autophosphrylation with PEP
HPr	General energy-coupling protein. Phosphorylated by EI~P at the N-1 position of a histidyl residue of HPr

Based on the phylogeny of the IIC proteins, the PTS transporters are divided into seven families that can taken up a wide range of monosaccharides, glycosides, polyols and disaccharides (Table 1.3) (Barabote and Saier, 2005; Saier, 2000). These PTS families can be grouped into three evolutionary distinct superfamilies/families, the mannose-fructose-sorbose family, the glucose-fructose-lactose superfamily and the ascorbate-galactitol superfamily. The glucose-fructose-lactose superfamily includes the glucose-glucoside, fructose-mannitol, lactose-N,N'-diacetylchitobiose-β-glucoside and glucitol families, while the ascorbate-galacticol superfamily comprises the galactitol and L-ascorbate families (Barabote and Saier, 2005).

Table 1.3 - The PTS families. Adapted from (Barabote and Saier, 2005).

Family	Description	Substrates[a]
4.A.1	The PTS Glucose-Glucoside Family	Glucose, Glucosamine, N-acetylglucosamine, Maltose
4.A.2	The PTS Fructose-Mannitol Family	Fructose, Mannose, Mannitol
4.A.3	The PTS Lactose-N,N'-Diacetylchitobiose-β-glucoside Family	Lactose, aromatic β-glucosides, Cellobiose, N,N'-diacetylchitobiose
4.A.4	The PTS Glucitol Family	Glucitol
4.A.5	The PTS Galactitol Family	Galactitol, D-arabitol
4.A.6	The PTS Mannose-Fructose-Sorbose Family	Glucose, Mannose, Fructose, Sorbose, N-acteylmannosamine
4.A.7	The PTS L-Ascorbate Family	L-ascorbate

[a] – some substrates used for each PTS family.

The genomic analysis of PTS permeases encoded in the genomes of 77 bacterial species showed the following order of prevalence of the PTS families: glucose-glucoside > fructose-mannitol > mannose-fructose-sorbose > lactose-N,N'-diacetylchitobiose-β-glucoside > L-ascorbate > galactitol > glucitol (Barabote and Saier, 2005). The PTS systems are abundant in LAB, being the main sugar transporters in these organisms.

1. 4 Glucose transport systems and metabolism in *L. lactis*

Sugar transporters have been studied in *L. lactis* for more than 40 years (Citti *et al.*, 1967; Kandler, 1983; McKay *et al.*, 1969; Thompson, 1987). *L. lactis* can import various sugars, either by PTS systems, ABC transporters or secondary carriers (Table 1.4). Nevertheless, the PTS are by far the most abundant transport system in this organism. This observation is corroborated by a genomic analysis of *L. lactis* subsp. *cremoris* SK11 showing that half of the sugar transporters are PTS, 32% are ABC transporters and 18% are secondary carriers (Lorca *et al.*, 2007).

Table 1.4 - Sugar transport systems of *L. lactis.* Adapted from (Castro, 2009).

Transporter	Genes	Reference
PTS		
Lactose-PTS	*lacFE*	(de Vos *et al.*, 1990; Thompson, 1979)
Sucrose-PTS	*llmg_0453*	(Thompson and Chassy, 1981)
Trehalose-PTS	*trePP*	(Andersson *et al.*, 2001)
Galactose-PTS		(Thompson, 1980)
Fructose-PTS	*fruA*	(Barriere *et al.*, 2005)
Mannitol-PTS	*mtlARFD*	(Bolotin *et al.*, 2001)
Mannose-PTS	*ptnABCD*	(Pool *et al.*, 2006; Thompson *et al.*, 1985)
Cellobiose-PTS	*celB, ptcBA*	(Kowalczyk *et al.*, 2008)
Cellobiose-PTS	*ptcBAC*	(Pool *et al.*, 2006)
Secondary carriers		
Xylose: H^+ symporter	*xylT*	(Bolotin *et al.*, 2001)
Galactose permease	*galP*	(Grossiord *et al.*, 2003; Thompson, 1980)
Glucose permease	*glcU*	(Castro *et al.*, 2009)
ABC		
Maltose-ABC	*malEFG*	(Andersson and Radstrom, 2002; Law *et al.*, 1995)
Ribose-ABC	*rbsBCD*	(Bolotin *et al.*, 2001; Wegmann *et al.*, 2007)

Glucose is reasonably cheap, abudant in nature, can be found either as free monosaccharide or as constituent of structural and reserve polysaccharides, and has been the substrate of choice for most bacteria used in biotechnological applications since it allows high biomass yields and growth rates. Besides being the preferred sugar of many model organisms, including *L. lactis,* glucose can also repress the utilization of other carbon sources in a process called carbon catabolite repression (Castro *et al.*, 2009; Gorke and Stulke, 2008). Glucose metabolism in *L. lactis* has been extensively studied in the past 30 years, however just very recently was possible to unravel all the routes for glucose uptake in this bacterium (Castro *et al.*, 2009).

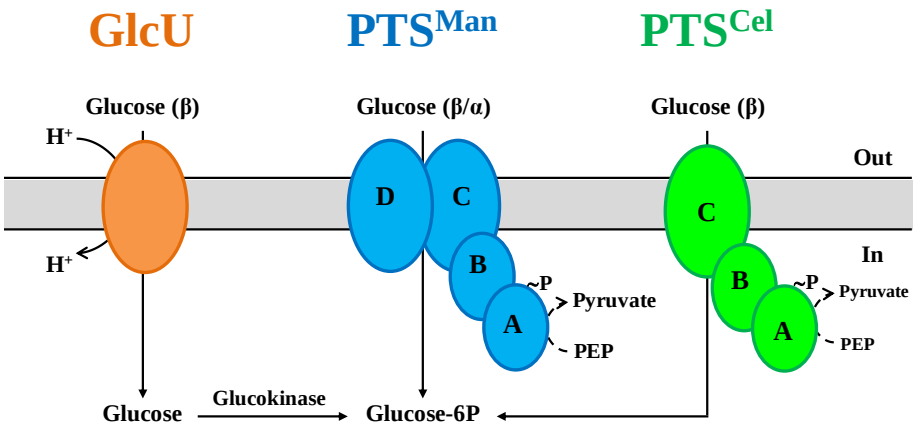

Fig. 1.4 – Schematic overview of glucose transporters in *L. lactis*.

L. lactis transports glucose via two PTS systems, PTS^{Man} and the cellobiose-PTS (PTS^{Cel}), and a non-PTS permease (GlcU), that belongs to the group of secondary carriers (Fig. 1.4) (Castro *et al.*, 2009; Pool *et al.*, 2006; Thompson and Chassy, 1985). The PTS^{Man} is the main sugar transporter in *L. lactis* and is encoded by the *ptnABCD* operon. In 2006, Pool and co-workers identified the PTS^{Cel} as an alternative glucose transporter in *L. lactis*; the soluble protein complex $EIIBA^{Cel}$ is encoded by *ptcB* and *ptcA* genes and the *ptcC* gene encodes the membrane-associated $EIIC^{Cel}$ domain (Pool *et al.*, 2006). Both PTSs transport glucose with high capacity, but contrary to PTS^{Man}, the PTS^{Cel} exhibits low affinity for glucose, suggesting that this sugar is not its principal substrate. More recently, GlcU, that operate by H^+ symport, was identified as the sole glucose non-PTS permease in *L. lactis*. This system has a moderate capacity and a low affinity for glucose uptake (Castro *et al.*, 2009). In addition to its high capacity and affinity for glucose, PTS^{Man} is also the only system that efficiently transports both α- and β-glucose, while the other two systems just transport β-glucose.

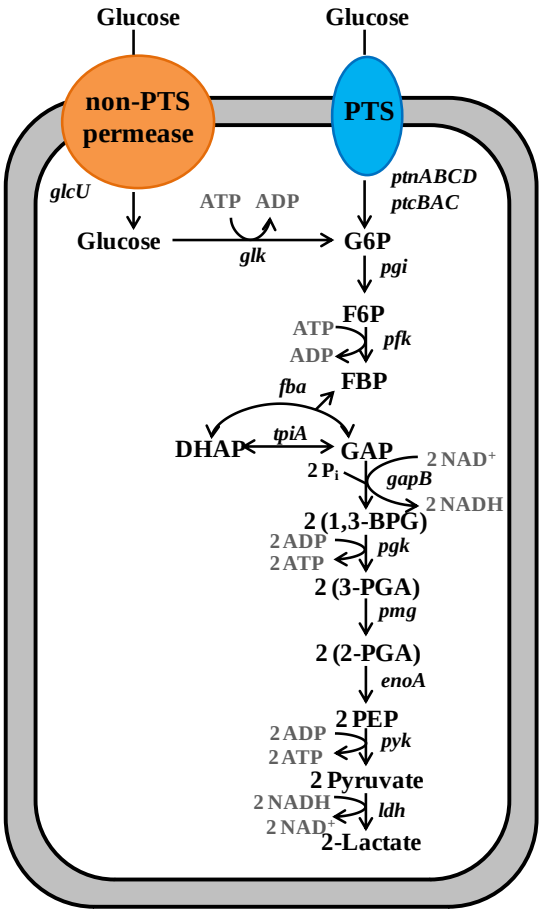

Fig. 1.5 - Schematic overview of glucose fermentation in *L. lactis*. The reactions are catalyzed by the following enzymes: PTSs (*ptnABCD* and *ptcBAC*), non-PTS permease (*glcU*), glucokinase (*glk*), phosphoglucose isomerise (*pgi*), 6-phosphofructokinase (*pfk*), fructose 1,6-biphosphate aldolase (*fba*), triosephosphate isomerise (*tpiA*), glyceraldehyde 3-phosphate dehydrogenase (*gapB*), phosphoglycerate kinase (*pgk*), phosphoglyceromutase (*pmg*), enolase (*enoA*), pyruvate kinase (*pyk*), lactate deydrogenase (*ldh*). Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-biphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; BPG, 1,3-biphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phospho*enol*pyruvate. Adapted from (Pool, 2008).

L. lactis is a homofermentative LAB that converts glucose to pyruvate via the EMP pathway. The enzymatic reactions involved in the fermentation of glucose by this pathway are shown in Fig. 1.5. Glucose transported by the PTSs enters directly in the glycolysis as glucose 6-phosphate (G6P), however, the sugar transported by GlcU has to be intracellularly phosphorylated by glucokinase (*glk*) (Castro *et al.*, 2009). The G6P is converted to fructose 1,6-biphosphate (FBP) by the action of phosphoglucose isomerase and 6-phosphofructokinase. Subsequently, FBP is converted into dihydroxyacetone phosphate (DHAP) and glyceraldehdyde 3-phosphate (GAP) by the fructose 1,6-biphosphate aldolase. These triose-phosphates can be interconvert by triosephosphate isomerase. The GAP molecule is converted to 3-phosphoglycerate (3-PGA) by the sequential action of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. After that, phosphoglyceromutase and enolase

transform 3-PGA into PEP. In a final stage, PEP is converted to pyruvate by pyruvate kinase. Being an homofermentative organism, *L. lactis* will convert almost all the pyruvate into to lactate by the action of LDH. Nevertheless, under conditions of limited glucose availability or when it has to metabolize less favourable sugars, the metabolism can shift to a mixed-acid fermentation profile and pyruvate is converted into a mixture of lactate, acetate, ethanol, formate, diacetyl, acetoin or 2,3-butanediol (Fig. 1.6) (Cocaign-Bousquet *et al.*, 2002; Jensen *et al.*, 2001; Melchiorsen *et al.*, 2001).

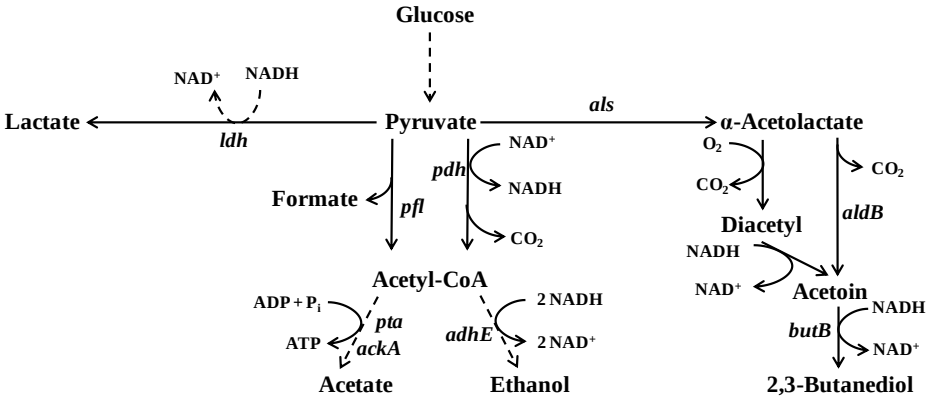

Fig. 1.6 - Schematic overview of the mixed-acid fermentation in *L. lactis*. Abbreviations: *ldh*, lactate dehydrogenase; *als*, α-acetolactate synthase; *pfl*, pyruvate formate lyase; *pdh*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *ackA*, acetate kinase; *adhE*, acetaldehyde:alcohol dehydrogenase; *aldB*, α-acetolactate decarboxylase; *butB*, 2,3-butanediol dehydrogenase. Adapted from (Pool, 2008).

1. 5 Production of succinate

In the last century the chemical industry has been interconnected with the oil industry. However, the escalating oil prices and environmental awareness has conducted the research efforts for alternative biotechnological routes for production of high-value compounds, such as succinate (Cox *et al.*, 2006; Hermann *et al.*, 2007; Lin *et al.*, 2005a; Litsanov *et al.*, 2012b).

Succinic acid is a member of the C4-dicarboxylic acid family with a wide range of applications in food (can be used as additives or flavoring agent), pharmaceutical, polymer, detergent and agricultural industries (Zeikus *et al.*, 1999). This compound is usually produced by chemical synthesis, but the high costs associated with this production process have strengthened the search for a biotechnological process of succinate production from renewable carbon sources with high yields. (Litsanov *et al.*, 2012a; Wang *et al.*, 2011). In fact, the biotechnological-derived succinate was identified as one of the top 12 value-added chemicals that could be produced in large quantities from biomass (Litsanov *et al.*, 2012b).

Succinate is a metabolite that can be formed in bacteria under both anaerobic and aerobic conditions (Fig. 1.7).

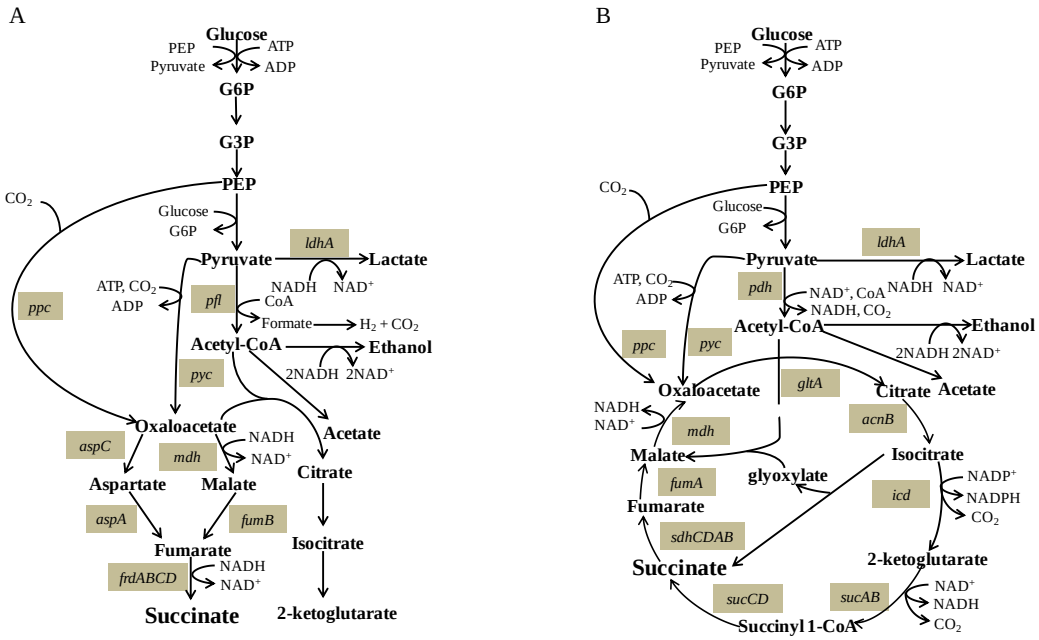

Fig. 1.7 - Major anaerobic (A) and aerobic (B) pathways for succinate production. The reactions are catalyzed by the following enzymes: lactate dehydrogenase A (*ldhA*), pyruvate formate lyase (*pfl*), phospho*enol*pyruvate carboxylase (*ppc*), pyruvate carboxylase (*pyc*), aspartate aminotransferase (*aspC*), malate dehydrogenase (*mdh*) aspartate ammonia-lyase (*aspA*), fumarase B (*fumB*), fumarate reductase (*frdABCD*), pyruvate dehydrogenase (*pdh*), citrate synthase (*gltA*), aconitate hydratase 2 and 2-methylisocitrate dehydratase (*acnB*), isocitrate dehydrogenase (*icd*), succinyl coenzyme A synthetase (*sucAB*), succinyl coenzyme A synthetase (*sucCD*) and succinate dehydrogenase (*sdhCDAB*). Abbreviations: G6P, glucose 6-phosphate; G3P, glucose 3-phosphate; PEP, phospho*enol*pyruvate. Adapted from (Cox *et al.*, 2006).

In bacteria such as *E. coli*, *Corynebacterium glutamicum* or *L. lactis*, succinate is a minor product of mixed-acid fermentation in anaerobic conditions (Litsanov *et al.*, 2012b). Under these conditions, succinate is formed from PEP and/or pyruvate by a carboxylation reaction and a subsequent series of reductive steps (Fig. 1.7A). More specifically, in the conversion of PEP to succinate, PEP is carboxylated to oxaloacetate (OAA) through CO_2 fixation, in a reaction catalyzed by phospho*enol*pyruvate carboxylase (*ppc*). Additionally, pyruvate is converted to OAA by pyruvate carboxylase (*pyc*) through CO_2 fixation in an ATP-consuming reaction. After that, OAA is converted to malate or aspartate, that are subsequently converted to succinate. In this process, 2 mol of NADH are oxidized to 2 mol of NAD^+. Since only 2 mol of NADH are formed per mole of glucose converted to PEP or pyruvate, the maximum yield for succinate production under anaerobic conditions is one mol of succinate/mol of glucose consumed (Cox *et al.*, 2006; Lin *et al.*, 2005c).

Under aerobic conditions, succinate is just an intermediate of the tricarboxylic acid (TCA) cycle (Fig 1.7B). *E. coli*, *Saccharomyces cerevisiae* or *C. glutamicum* have been engineered for increased succinate production under aerobic conditions. The key mutation in this process is the deletion of the succinate dehydrogenase genes, which leads to a high accumulation of succinate as well as acetate. Subsequent deletion of all the pathways producing

acetate results in a strong reduction of acetate levels and improvement of succinate production. The production can be optimized with the limitation of biomass formation, since is provided more carbon for succinate production (Litsanov *et al.*, 2012b).

A common strategy in succinate production processes is the increase of the PEP potential in the biocatalysts. For that, the PEP-dependent sugar transport, i.e. PTS, is frequently disrupted and replaced by non-PTS transporters. In addition, inactivation of other PEP consuming pathways in the glycolysis and TCA cycle are frequently considered (Cabrera-Valladares *et al.*, 2012; Escalante *et al.*, 2012; Gosset, 2005; Lin *et al.*, 2005c). Another intermediate in succinate production often targeted is OAA. This metabolite can be formed by carboxylation of PEP or pyruvate. In order to increase OAA levels, native or heterologous, PEP and pyruvate carboxylases are overproduced in succinate producing organisms (Lin *et al.*, 2005b; Litsanov *et al.*, 2012b).

1. 6 Specific goals of this work

As mentioned above, *L. lactis* possesses three glucose uptake systems. These transporters have been individually characterized in Castro *et al.* 2009. However, in the earlier study the contribution of each transporter to the overall glucose metabolism was not addressed. To this end, three single deletion mutants (Δ*ptnABCD*, Δ*glk* and Δ*ptcBA*) were constructed and characterized. During inactivation of *glk* and *ptcBA*, but not *ptnABCD*, two types of colonies were isolated. Of each transporter mutant, one type of colony (herein denominated isolates 1) displayed only a modest effect on glucose metabolism, while the other (isolates 2) was drastically affected. The isolate 2 of Δ*glk* showed reduced uptake rate (V_{max}) and affinity for glucose, whereas the isolate 2 of Δ*ptcBA* was characterized by a lower V_{max} (Table 1.5).

Table 1.5 – Kinetic properties of glucose transport in whole cells of *L. lactis* NZ9000 and its glucose-transport mutants. Adapted from (Pool, 2008).

Strains	***V*max (μmol min^{-1} mg prot^{-1})[a]**	***K*m (mM)[a]**	**Transporters present**
NZ9000	0.19±0.01	(5.2±0.2)*10^{-3}	PTS^{Man}, PTS^{Cel}, GlcU
Δ*ptnABCD*	0.17±0.01	5.17±0.37	PTS^{Cel}, GlcU
Δ*glk* (isolate 2)	0.22±0.03 0.01±0.00	4.81±0.25 (20±2.3)*10^{-3}	PTS^{Man}, PTS^{Cel}, GlcU^{+}
Δ*ptcBA* (isolate 2)	0.09±0.01	(3.5±0.0)*10^{-3}	PTS^{Man}, GlcU

[a] – Values of two independent experiments were averaged and are reported±SD of the two measurements. *V*max and *K*m were determined using the following glucose concentrations: NZ9000, 0.1 μM to 25 mM; *ΔptnABCD*, 0.5 μM to 10 mM; *Δglk* (isolate 2), 0.1 μM to 10 mM; *ΔptcBA* (isolate 2), 0.1 to 500 μM.
+ - The GlcU/Glk pathway for glucose dissimilation is blocked due to inactivation of *glk*.

Additionally to modifications in the kinetics properties of glucose transport, a substantial anomeric preference was observed in resting cells of isolate 2 of Δ*glk* that is accompanied by a clear reduction in glucose consumption rate, as determined by *in vivo* nuclear magnetic resonance (NMR) (compare panels C and D of Fig. 1.8). A reduction in glucose consumption rate also occurs in isolate 2 of Δ*ptcBA* but was not detected any preference for one of the glucose anomers (compare panels F and G of Fig. 1.8). As the permease/Glk and the PTS^{Cel} were expected to play a minor role we suspected that isolates 2 had acquired additional spontaneous mutations. Indeed, sequence analysis of the *ptn* operon in isolates 2 of Δ*glk* and Δ*ptcBA* identified single point-mutations in the genes codifying for the permease domain EIICD of the PTS^{Man} that were absent in isolates 1. In particular, these point-mutations translate into a G111::S111 change in the $EIIC^{Man}$ of Δ*glK* (isolate 2) and an M105::I105 mutation in the $EIID^{Man}$ of Δ*ptcBA* (isolate 2).

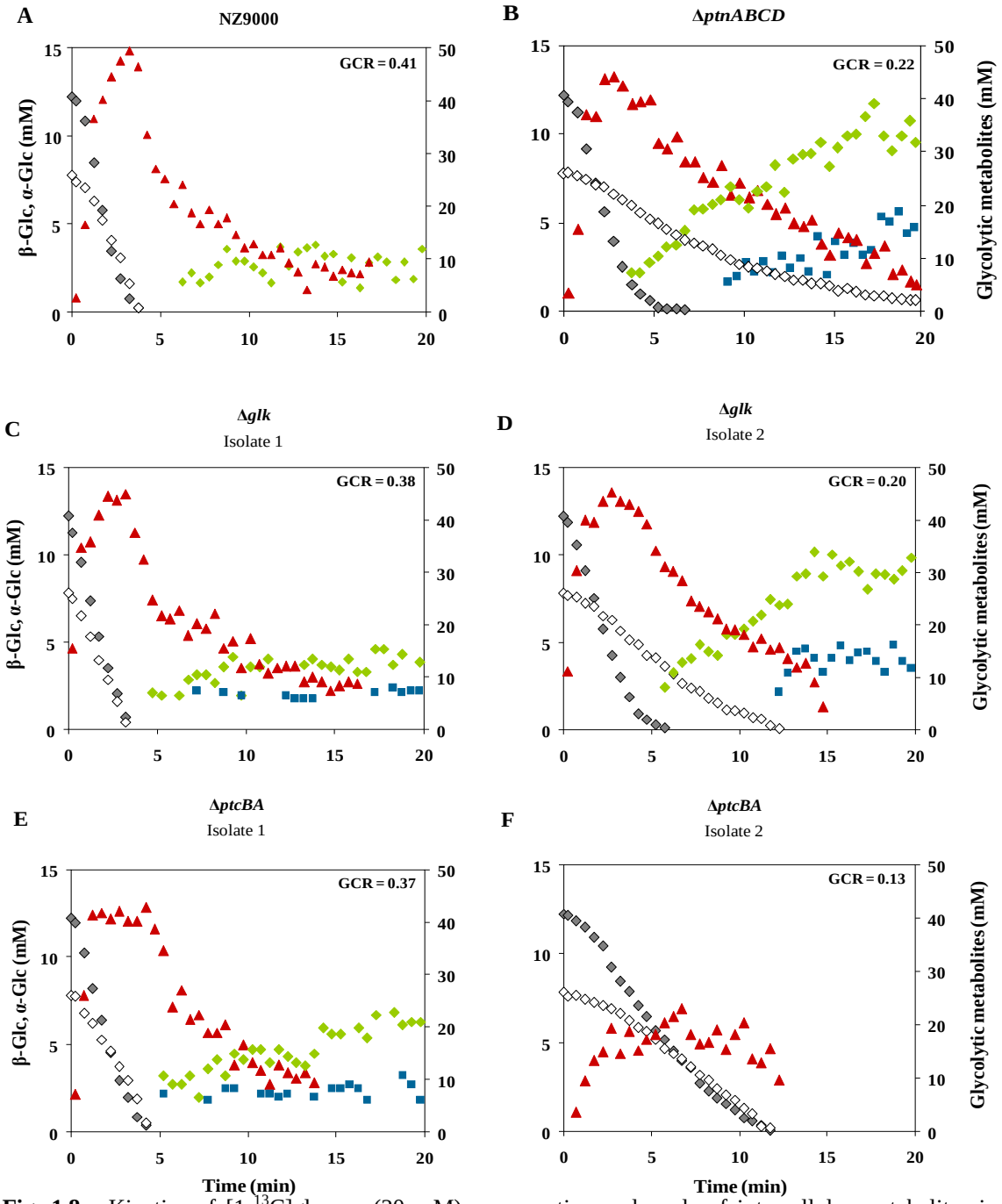

Fig. 1.8 - Kinetics of [1-^{13}C]glucose (20 mM) consumption and pools of intracellular metabolites in resting cells of *L. lactis* strain NZ9000 (A), Δ*ptnABCD* (B), Δ*glk* (isolate 1) (C), Δ*glk* (isolate 2) (D), Δ*ptcBA* (isolate 1) (E) and Δ*ptcBA* (isolate 2) (F), under anaerobic conditions at 30ºC with pH controlled at 6.5. Maximum glucose consumption rates (µmol min^{-1} mg prot^{-1}) are in the upper-right corners. Symbols: β-glucose (◆), α-glucose (◇), fructose 1,6-biphosphate (▲), 3-phosphoglycerate (◆), phospho*enol*pyruvate (■). Data of NZ9000, Δ*ptnABCD*, Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) was adapted from (Castro, 2009). Data of Δ*glk* (isolate 2) and Δ*ptcBA* (isolate 2) was adapted from (Pool, 2008).

The main goal of this work was to ascertain if the random mutations identified in isolates 2 conferred the observed phenotypes. Therefore, the single base changes identified in *ptnC* (coding for $EIIC^{Man}$) and *ptnD* (coding for $EIID^{Man}$) genes were introduced in the genome of *L. lactis* NZ9000 (wild-type), Δ*glK* (isolate 1) and Δ*ptcBA* (isolate 1) by directed mutagenesis.

A major metabolic consequence of glucose transport disruption is an improved PEP/pyruvate potential, and that was not an exception in the glucose transport mutants reported here. This led us to surmise a beneficial role of the PTS mutations in the development of lactococcal hosts for the production of chemicals requiring a carboxylation step, such as succinate. The latter compound is detected as a minor metabolite of glucose metabolism in *L. lactis*. Thus, to test our hypothesis and as a second aim of this work, we considered overexpress PEP and/or pyruvate carboxylases genes in adequate lactococcal strains.

2. Material and Methods

2. 1 Bacterial strains and growth conditions

Strains and plasmids used in this study are shown in Table 2.1. *Escherichia coli* DH5α was grown in Luria-Bertani medium (LB) (Bertani, 1951) at 37ºC. *L. lactis* strains were routinely cultivated at 30ºC in M17 medium (Difco™, Sparks, MD, USA) supplemented with 0.5% glucose (w/v) (GM17), and bacterial stocks kept at -80ºC in 20% (v/v) glycerol. In order to keep uniformity between cultures, working stocks were prepared by growing the cells up to the exponential phase in rubber stoppered bottles containing 25 mL GM17, followed by the addition of glycerol to a final concentration of 20% (v/v) and storage at -80ºC in 1 mL aliquots. For physiological studies, *L. lactis* strains were grown at 30ºC without pH control (initial pH of 6.5) in rubber stoppered bottles containing Chemically Defined Medium (CDM) supplemented with disodium β-glycerophosphate to buffer the cultivations and 1% glucose (w/v) (Neves *et al.*, 2002). Cultures were initiated by addition of a pre-culture, which was grown overnight for 14 h under anaerobic conditions in glucose-CDM, to an initial optical density at 600 nm (OD_{600}) of about 0.05. Antibiotic selection was used when appropriate: in *E. coli* 150 mg/L erythromycin; in *L. lactis* 2.5 mg/L erythromycin and 5 mg/L chloramphenicol. For controlled overproduction of *ppc* using the nisin inducible controlled expression (NICE) system for expression, nisin (1 μg/L) was added at an OD_{600} of about 0.3 and the cultures were harvested after 2h of nisin induction. Growth was monitored by measuring OD_{600} every hour. Specific growth rates (μ) were calculated through linear regressions of the plots of natural logarithm (ln) versus time during the exponential growth phase. The generation time (t/n) was calculated using the formula $\frac{ln2}{\mu}$.

Table 2.1 – Strains and plasmids used in this study.

	Description	**Reference**
Strains		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
NZ9000	MG1363 derivative carrying *pepN*::*nisRK*	(Kuipers *et al*., 1998)
Δ*ptnABCD*	Derivative of NZ9000 containing a 1736-bp deletion in *ptnABCD*	(Pool *et al*., 2006)
Δ*glk* (isolate 1)	Derivative of NZ9000 containing a 404-bp deletion in *glk*	(Castro, 2009)
Δ*glk* (isolate 2)	Derivative of NZ9000 containing a 404-bp deletion in *glk* and a spontaneous G111::S111 mutation in $EIIC^{Man}$	(Pool, 2008)
Δ*ptcBA* (isolate 1)	Derivative of NZ9000 containing a 657-bp deletion in *ptcBA*	(Castro, 2009)
Δ*ptcBA* (isolate 2)	Derivative of NZ9000 containing a 657-bp deletion in *ptcBA* and a spontaneous M105::I105 mutation in $EIID^{Man}$	(Pool, 2008)
NZ9000*ptnD'*	Derivate of NZ9000 containing a directed M105::I105 mutation in $EIID^{Man}$	This work
NZ9000*ptnC'*	Derivate of NZ9000 containing a directed G111::S111 mutation in $EIIC^{Man}$	This work
Δ*glkptnC'*	Derivate of Δ*glk* (isolate 1) containing a directed G111::S111 mutation in $EIIC^{Man}$	This work
Δ*ptcBAptnD'*	Derivate of Δ*ptcBA* (isolate 1) containing a directed M105::I105 mutation in $EIID^{Man}$	This work
NZ9000*ppc*$^+$	Derivate of NZ9000 carrying pNZ8048*ppc*$^+$	This work
Plasmids		
pCS1966	Em^r, harbors *oroP* gene	(Solem *et al*., 2008)
pCS1966/*ptnC'*	Em^r, derivate from pCS1966 specific for integration in *L. lactis* containing a G111::S111 mutation in the *ptnC* gene	This work
pCS1966/*ptnD'*	Em^r, derivate from pCS1966 specific for integration in *L. lactis* containing a M105::I105 mutation in the *ptnD* gene	This work
pNZ8048	Cm^r, nisin inducible P*nisA*	(de Ruyter *et al*., 1996)
pNZ8048*ppc*$^+$	Cm^r, derivate from pNZ8048 with *ppc* (*Corynebacterium glutamicum* ATCC13032) cloned in the *Sph*I/*Xba*I site	This work

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

2. 2 Molecular techniques

General molecular techniques were performed as described by Sambrook *et al.* (1989). Chromosomal DNA was isolated from *L. lactis* strains according to Johansen and Kibenich

(1992). Plasmid DNA isolation was carried out using the High Pure Plasmid Isolation Kit (Roche Applied Science, Mannheim, Germany) with the following modifications for *L. lactis*: bacterial cell pellet was re-suspended in Birnboim A solution (Birnboim, 1983), containing 5 mg/mL lysosyme and 30 µg/mL RNaseI, and incubated at 55ºC for 10-15 min before addition of Buffer P2. *L. lactis* was transformed with plasmid DNA by electroporation (Holo and Nes, 1995). Restriction enzymes and T4 DNA ligase (New England Biolabs, Ipswich, MA, USA), Taq DNA polymerase and Pwo DNA polymerase (Bioline or Bioscience) were used according with the recommendations provided by the suppliers. Primers (listed in Table 2.2) were purchased from Stab Vida (Portugal) and Metabion (Germany). The polymerase chain reactions (PCR) were performed in the MyCycler TM thermal cycler (Bio-Rad, Hercules, CA, USA). Purification of PCR products was done using the High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany). Sequencing of DNA fragments was performed by Stab Vida (Stab Vida, Portugal).

Table 2.2 – Primers used in this work.

Primers	Sequence (5' to 3')	Restriction site
ptnC_Gly111_KO1	GCTCTAGAGCAACAATCGAAGGTGGTGTACC	*Xba*I
ptnC_Gly111_KO2	CAATACAAGACTAGCAGTTGCAAGCAAGATAGCA	
ptnC_Gly111_KO3	GCAACTGCTAGTCTTGTATTGACTACTCTTGTAC	
ptnC_Gly111_KO4	CCGCTCGAGGGAGCAGCAACGTATGGGTGAG	*Xho*I
ptnD_Met105_KO1	GCTCTAGAGGTTTGCGTATCGCTATCCCTGC	*Xba*I
ptnD_Met105_KO2	AAGAGGACCCATTATACCAACTTTTACCCCTTGA	
ptnD_Met105_KO3	GTAAAAGTTGGTATAATGGGTCCTCTTGCCGGTA	
ptnD_Met105_KO4	CCGCTCGAGCATGATTTGAGCCCAACGACCG	*Xho*I
pCS1966_cPCR_Forw	ACTCGCAATTAAGCGAGTTGG	
pCS1966_cPCS_Rev01	ATGCTTCCGGCTCGTATGTTGTGTGG	
MAMA_ptnC111	CAAGAGTAGTCAATACAAGATT	
MAMA_ptnD105	CCGGCAAGAGGACCCATTGT	
PciI-pyc-for	CGACATGTCGACTCACACATCTTC	*Pci*I
BspHI-pyc-for	GGCGCGTCATGAGCACTCACACATCTTC	*Bsp*HI
SphI-pyc-rev	ACATGCATGCTTAGGAAACGACGACGATC	*Sph*I
SphI-ppc-for	ACATGCATGCTTAATAAAGGAGGAATAATAATGACTGA TTTTTTACGCGATG	*Sph*I
XbaI-ppc-rev	GCTCTAGACTAGCCGGAGTTGCGCAGCG	*Xba*I
Pnis-Fw	GTCGATAACGCGAGCATAATAAACG	
Pnis-Rv	CGTGCTGTAATTTGTTTAATTGCC	

Underlined sequences indicate the respective restriction site.

2. 3 Construction of mutant strains and plasmids

Point-mutations - *L. lactis* NZ9000, Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) mutants carrying point-mutations in *ptnC* and/or *ptnD* genes were constructed by combining site-directed mutagenesis and a system to introduce unmarked mutations (Fig. 2.1). For that the chromosomal integration vector pCS1966 was employed, allowing the positive selection of the cells in which the plasmid has been excised from the genome (Solem *et al.*, 2008). To create the point-mutation G111::S111 in *ptnC* gene, two DNA fragments were amplified from *L. lactis* MG1363 (Gasson, 1983) genomic DNA using the primer pairs ptnC_Gly111_KO1/ ptnC_Gly111_KO2 and ptnC_Gly111_KO3/ ptnC_Gly111_KO4. These oligonucleotides were designed to generate PCR products containing complementary end sequences and a single base substitution ggt::agt in codon 111 of *ptnC*. Both flanking regions were then spliced by overlap extension in a second PCR reaction as described elsewhere (Ho *et al.*, 1989) and cloned as *Xba*I/*Xho*I restriction fragment in pCS1966 (Solem *et al.*, 2008), resulting in plasmid pCS1966/*ptnC*'. The plasmid was obtained and maintained in *E. coli* DH5α. The same strategy was pursued to construct the vector to generate a M105::I105 point mutation in *ptnD*, using the primer pairs ptnD_Met105_KO1/ptnD_Met105_KO2 and ptnD_Met105_KO3/ptnD_Met105_KO4, originating the plasmid pCS1966/*ptnD*'. In this case the primers ptnD_Met105_KO2 and ptnD_Met105_KO3 were designed to generate PCR products containing complementary end sequences and a single base substitution atg::ata in codon 105 of *ptnD*. The vector pCS1966/*ptnC*' was introduced into *L. lactis* NZ9000 and Δ*glk* (isolate 1) whereas plasmid pCS1966/*ptnD*' was introduced in strains NZ9000 and Δ*ptcBA* (isolate 1) via electroporation. Selection of integrants was done on GSM17-agar plates with 2.5 mg/L erythromycin. Confirmation of chromosomal integration of the constructs was done by PCR using diferent primers combinations.

A two-step homologous recombination event was induced by growing cells on selective CDM without bases or SA medium plates (Jensen and Hammer, 1993) suplemmented with 20 µg/mL of 5-fluoroorotic acid hydrate. This technique allows an efficient and quick positive survival strategy to monitor both integration and excision of the vector backbone from the chromosome, enabling the production of unmarked strains in an easy and faster way (Pinto *et al.*, 2011b). Colonies with the complete, but mutated gene were selected by Mismatch Amplification Mutation Assay (MAMA) PCR analysis (see below) and the point-mutation confirmed by DNA sequencing of the respective genes in mutant strains.

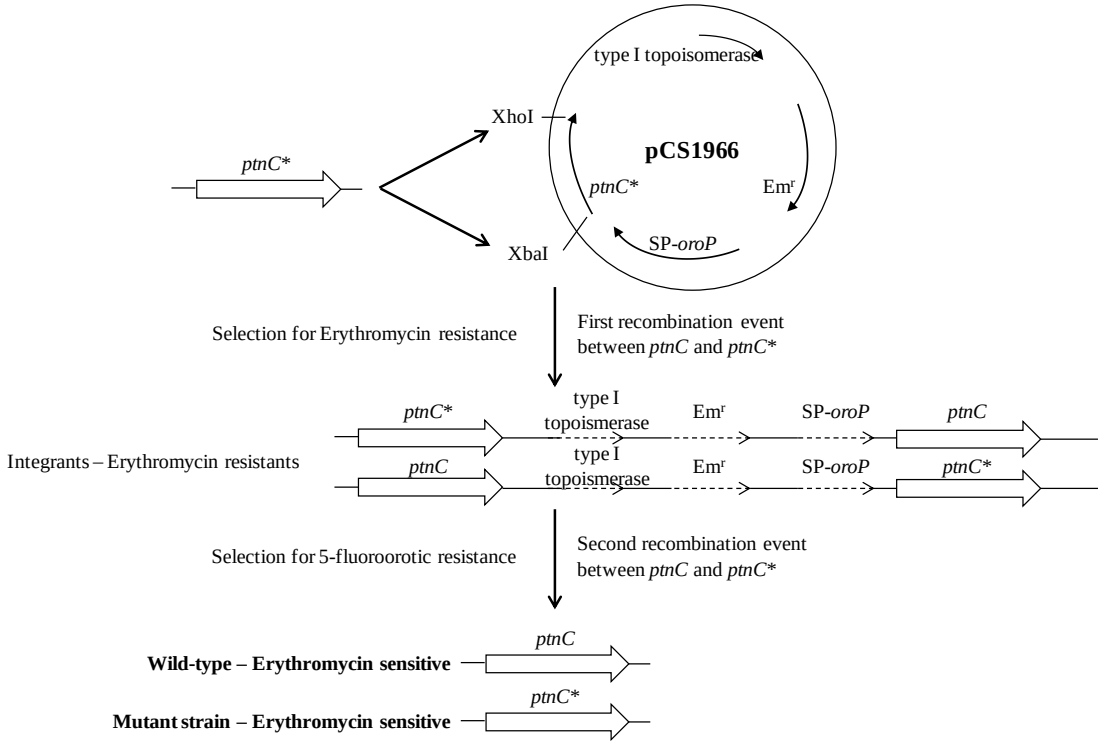

Fig. 2.1 – Representative scheme for exploitation of the *oroP*-based selection/conteurselection vector pCS1966 for construction of strains with a specific point-mutation in *ptnC* gene. *ptnC** has a single base substitution (ggt::agt) in codon 111 of *ptnC* gene. This system was also used to generate strains with specific point-mutations in *ptnD* gene.

Overxpression of pyc and ppc genes - The overexpression of *pyc* and *ppc* genes was done in *L. lactis* NZ9000. To overexpress the pyruvate carboxylase activity in *L. lactis*, the *pyc* gene was obtained by PCR amplification from the vector pAN6-*pyc*^{P458S}*ppc* (a derivative from pAN6 containing the *pyc*^{P458S} gene from *Corynebaterium glutamicum* DM1727 carrying a point-mutation in the native start codon from GTG to ATG and the *ppc* gene from *C. glutamicum* ATCC13032) (*Litsanov et al.*, 2012b) using either the primers PciI-pyc-for/SphI-pyc-rev or BspHI-pyc-for/SphI-pyc-rev. The resulting PCR product was digested with *Pci*I/*Sph*I or with *Bsp*HI/*Sph*I and ligated to the *Nco*I/*Sph*I sites of the nisin inducible expression vector pNZ8048 (Kuipers *et al.*, 1998). Unfortunately, after several attempts we failed to obtain NZ9000 transformants carrying the *pyc* gene cloned into pNZ8048 (see Results section).

To obtain the expression vector pNZ8048*ppc*$^{+}$, the *ppc* gene from *C. glutamicum* ATCC13032 was amplified from the vector pAN6-*pyc*^{P458S}*ppc* (*Litsanov et al.*, 2012b), using the oligonucleotides SphI-ppc-for/XbaI-ppc-rev. Besides the *Sph*I restriction site, the SphI-ppc-for primer introduced a consensus lactococcal ribosome binding site (5' TAAAGGAGG 3') and a 7-base spacer (5' AATAATA 3') (de Vos, 1987). The resulting PCR product was digested with *Sph*I/*Xba*I and cloned into the *Sph*I/*Xba*I sites of pNZ8048, yielding the plasmid pNZ8048*ppc*. This vector was obtained and mantained in strain NZ9000.

2. 4 Mismatch Amplification Mutation Assay (MAMA) PCR

The MAMA PCR allows specific amplification of sequences differing by as little as a single base pair. This technique presents some advantages: is rapid, simple and can readily be applied to analyze nanogram quantities of genomic DNA (Cha *et al.*, 1992). This technique was used to screen point-mutations in strains NZ9000*ptnC*', NZ9000*ptnD*', Δ*glkptnC*' and Δ*ptcBAptnD*'. Briefly, this PCR-based technique makes use of three primers: a paring primer A (ptnC_Gly111_KO1 or ptnD_Met105_KO1), the MAMA primer and the control primer (ptnC_Gly111_KO4 or ptnD_Met105_KO4) that anneals at a distal site (> 0.5 kb) on the same strand. The MAMA primers were designed to contain two mismatches with the wild-type allele and one mismatch with the mutant allele at the 3' end (Zeng and Burne, 2010). In a PCR reaction containing the three primers, the wild-type genotype originates a single band resulting from the annealing of paring primer A with the control primer, whereas one smaller band (from the annealing of paring primer A and MAMA primer) or two bands (from the annealing of paring primer A and MAMA primer plus paring primer A and control primer) are amplified from the mutant strain (Fig. 2.2).

L. lactis colonies resulting from the excision step were directly used in a 30 µL PCR reaction that contained 0.6 µM paring primer A, 0.4 µM MAMA primer and 0.2µM control primer. The PCR conditions were as follows: 1 cycle of 5 min at 95ºC, 30 cycles composed by 25 sec at 95ºC, 25 sec at 45ºC (*ptnD* point-mutation) or 55ºC (*ptnC* point-mutation) and 2 min at 72ºC, and 1 cycle of 2 min at 72ºC (adapted from the protocol described by Zeng and Burne, 2010).

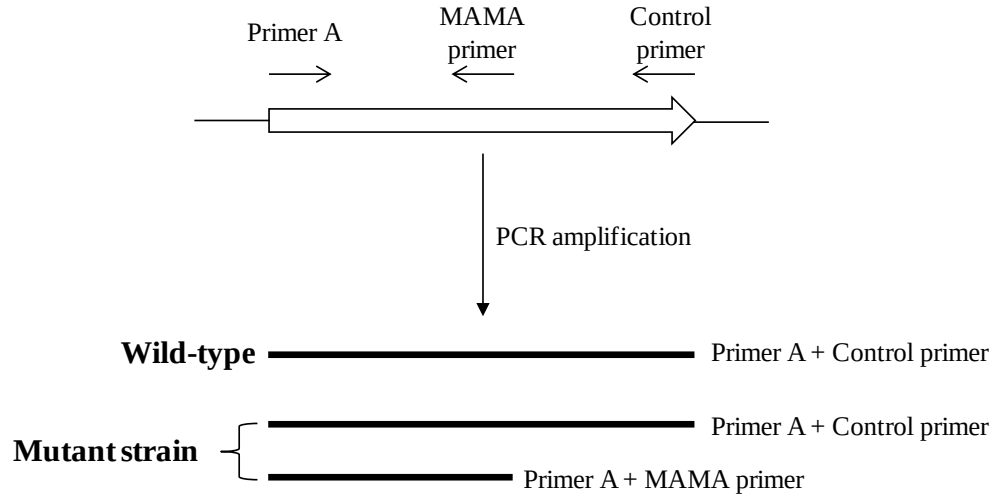

Fig. 2.2 – Schematic representation of the MAMA PCR result. Wild-type genotype originates a single band, whereas two bands are amplified in mutant strain.

2. 5 [^{14}C]glucose transport studies

Strains NZ9000, NZ9000*ptnC*', NZ9000*ptnD*', Δ*glk* (isolate 1), Δ*ptcBA* (isolate 1), Δ*glkptnC*' and Δ*ptcBAptnD*' were cultivated as batch cultures in 40 mL M17 medium supplemented with 1% glucose without pH control. Cells were harvested at mid-exponential phase of growth (5752 x *g*, 4ºC, 7 min), washed twice in 5 mM potassium phosphate-buffer (KP_i), pH 6.5 and suspended in the assay-buffer KP_i (50 mM, pH 6.5) to an OD_{600} of 4. The glucose uptake rates were essentially determined as described by Wolken *et al.* (2006). The cell suspension (50 µL) was pre-incubated for 2 min at 30ºC under conditions of constant stirring, and the reaction started with the addition of 50 µL of [U-^{14}C] glucose to a final concentration of 0.0005 to 15 mM (specific activity 0.02 – 19 µCi/µmol). Uptake was stopped at 5 or 10 seconds by the addition of 1.5 mL of ice-cold LiCl (0.1 M) immediately followed by filtering through a 0.45 µm-pore-size nitrocellulose filter (Millipore, Bedford, MA, USA). The filters were washed with 5 mL of KP_i (50 mM, pH 6.5), submerged in 5 mL of scintillation liquid cocktail (Sigma-Aldrich, Portugal) and the filter radioactivity was counted in a LS-6500 scintillation counter (Beckman Coulter, Fullerton, CA, USA). The background radioactivity was estimated by adding the radiolabeled glucose to the cell suspension immediately after the addition of 1.5 mL of ice-cold LiCl, followed by filtration (Wolken *et al.*, 2006). For kinetics analysis of glucose transport in Δ*ptcBAptnD*', the cell suspension was incubated with and without 2 mM of 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 nonlinear least squares regression analysis (Excel solver, Micrsoft Excel 2007) to the Michaelis-Menten equation. The transport assays were done in triplicate for all the strains, except for NZ9000 that was done only once.

2. 6 Quantification of fermentation products during growth

Cells were grown in CDM with 1% (w/v) glucose at 30ºC in rubber stoppered bottles without pH control (initial pH of 6.5), as described above. Samples (1.5 mL) were collected at time zero and at stationary phase of growth, centrifuged (16100 x *g*, 3 min, 4ºC), filtered through nylon membranes (0.22 µm), and the supernatant solutions were stored at -20ºC until analysis by high performance liquid chromatography (HPLC). Fermentation substrates and products were quantified by HPLC using a HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc.) and a refractive index detector (Shodex RI-101, Showa Denko K.K.) at 60ºC with 0.01N H_2SO_4 as the elution fluid and a flow rate of 0.5 mL min^{-1}. The Chromeleon® software was used for data treatment as described before (Gaspar *et al.*, 2004). A factor of 0.3605, determined from a

dry weight (DW, mg mL^{-1}) versus OD_{600} curve, was used to convert OD_{600} into dry weight (mg biomass mL^{-1}).

2. 7 *In vivo* ^{13}C-NMR experiments

The cell suspensions used in these experiments was prepared as follows: cells were grown in CDM containing 1% glucose (w/v) at 30ºC , without pH control (initial pH 6.5) until the mid-exponential phase of growth (OD_{600} 1.5), harvested by centrifugation (7519 x *g*, 7 min, 4ºC), washed twice with 5 mM KP_i and suspended to a final $OD_{600} \approx 80$ (protein 17 mg/mL) in 100 mM KP_i (pH 6.5) containing 6% D_2O and a drop of anti-foam. *In vivo* NMR experiments were done using a 10-mm NMR tube containing 3 mL of cell suspension. To avoid settling down of the cells and ensure an adequate supply of gases to the cell suspension an air-lift system was used inside the NMR tube (Santos and Turner, 1986). To make the system anaerobic, argon was bubbled through the air-lift system 10 minutes before and continuously after acquisition started. Glucose (15 mM) specifically labelled in carbon 1 was added at time 0 minutes and spectra acquired sequentially after its addition. The time course of glucose consumption, changes in pools of metabolites and product formation were monitored online and non-invasively. When all the substrate was consumed and there were no changes in the resonances of intracellular metabolites the acquisition was stopped and an NMR-extract was prepared as follows: 2.8 mL of cell suspension was transferred to a 10 mL beaker containing 280 µL of perchloric acid 6 M and incubated in an ice bath under vigourous stirring for 20 min, after which the cell extract was neutralized with 2 M KOH (pH 6.5-7.0) and the cell debris removed by centrifugation (45696 x *g*, 20 min, 4ºC). The supernatant (NMR-extract) was used for quantification of end products and minor metabolites as described below. Although individual experiments are illustrated in each figure, each type of *in vivo* NMR experiment was repeated at least twice and the results were highly reproducible. The values reported are averages of two experiments.

2. 8 Quantification of products by NMR

Lactate and acetate were quantified in NMR-extracts by ^{1}H-NMR (Neves *et al.*, 1999). Formic acid (sodium salt) was added to the samples and used as an internal concentration standard. The concentration of minor products (ethanol, alanine, 2,3-butanediol) and metabolic intermediates that remained inside the cells (pyruvate) was determined from the analysis of ^{13}C spectra of NMR-extracts as described by Neves *et al.* (1999). The concentration of labelled lactate determined by ^{1}H-NMR was used as a standard to calculate the concentration of the other metabolites in the sample.

2. 9 Quantification of intracellular metabolites in living cells by ^{13}C-NMR

Due to the fast pulsing conditions used for acquiring *in vivo* ^{13}C-spectra, correct quantification of the intracellular metabolites required the use of correction factors that allow the conversion of peak intensities into concentrations. Correction factors of 0.73±0.04 and 0.71±0.04 were used for resonances of fructose 1,6-bisphoshate (FBP); phospho*enol*pyruvate (PEP) or 3-phosphoglycerate (3-PGA) , respectively (Neves *et al.*, 2000). For pyruvate a correction factor of 1.43±0.54 was determined during this study. The quantitative kinetic data for intracellular metabolites were calculated from the areas of the relevant resonances, by applying the correction factors and comparing with the intensity of the lactate resonance in the last spectrum of the sequence. The lower limit for *in vivo* NMR detection of intracellular metabolites was 3-4 mM. Intracellular metabolite concentrations were calculated using a value of 2.9 µL mg^{-1} of protein for the intracellular volume of *L. lactis* (Poolman *et al.*, 1987).

2. 10 NMR Spectroscopy

Carbon-13 spectra were acquired at 125.77 MHz on a Bruker AVANCE II 500 MHz spectrometer (Bruker BioSpin GmbH). All *in vivo* experiments were run using a quadruple nuclei probe head at 30 °C as described elsewhere (Neves *et al.*, 1999). The acquisition parameters were as follows: spectral width, 30 kHz; pulse width, 9 µs (60º flip angle); data size, 32K; recycle delay, 1.5 s; number of transients, 20. Carbon chemical shifts are referenced to the resonance of external methanol designated at 49.3 ppm.

2. 11 Determination of phospho*enol*pyruvate carboxylase activity

Cells were grown in glucose-CDM until mid-exponential phase as described above, harvested by centrifugation (5752 x *g*, 7 min, 4ºC), washed twice with KP_i 5 mM (pH 6.5), resuspended in 9 mL of KP_i 50 mM (pH 6.5), aliquoted out in 3 mL portions and kept at -20ºC. Crude extracts were prepared by passage through a French press (three passages at 900 psi) and centrifugation for 30 min at16100 x *g* and 4ºC to remove cell debris and the supernant used for determination of enzymatic activity (Eikmanns *et al.*, 1989).

The PPC activity was assayed as described by Eikmanns *et al.* (1989) in a 1 mL reaction mixture containing 100 mM Tris-HCl (pH 6.5); 5 mM PEP; 10 mM $MgSO_4$; 10 mM $NaHCO_3$; 0.15 mM NADH, 0.1 mM dithiothreitol (DTT) and 10 U of malate dehydrogenase (MDH). The reaction was performed at 30ºC in a Beckman Coulter DU® 800 spectrophotometer. The rate of NADH consumption was measured spectrophotometrically at 340 nm. One unit of enzyme

activity is the amount of enzyme catalyzing 1 µmol substrate per minute under the experimental conditions used.

2. 12 Chemicals

[1-^{13}C]glucose (99% enrichment) and [U-^{14}C]glucose (310 mCi/mmol) were obtainted from Campro Scientific (Veenendaal, The Netherlands) and Amersham Biosciences (GE Healthcare, Europe), respectively. All other chemicals were reagent grade.

3. Results

3. 1. Insertion of point-mutations on *ptnC* and *ptnD* genes

The work herein presented originated from the realization that directed strain construction, in particular during inactivation of glucose-transporters in *L. lactis*, promoted the occurrence of additional spontaneous mutations.

Indeed, sequencing of the *ptn* operon from two different colonies of Δ*glk* and Δ*ptcBA* strains (Fig. 3.1) identified single point-mutations in the genes codifying for the permease domain EIICD of the PTS^{Man} in isolates 2 that were absent in isolates 1 of the same strains. In particular, a single base change in codon 111 (ggt::agt) of *ptnC* was identified in Δ*glK* isolate 2 which translates in a substitution of Gly by a Ser in $EIIC^{Man}$. In isolate 2 of Δ*ptcBA* was identified a Met105::Ile105 mutation in the $EIID^{Man}$ also resulting from a single base change in codon 105 (atg::ata) of *ptnD* gene. To assess if these spontaneous point-mutations correlate with the different metabolic profiles and kinetic properties of glucose uptake observed between both isolates of Δ*glk* and Δ*ptcBA* mutants (Fig. 1.8 and Table 1.5), the specific single base changes were introduced in *ptnC* and *ptnD* genes of isolates 1 by site-directed mutagenesis (see Material and Methods section for details) originating strains Δ*glKptnC'* and Δ*ptcBAptnD'* (Table 2.1), respectively. In parallel, the *ptnC* and *ptnD* point-mutations were also introduced in the wild-type strain NZ9000 originating strains NZ9000*ptnC'* and NZ9000*ptnD'*, respectively. These strains will allow ascertaining if the substitutions alone have an effect on the glucose transport and metabolism of *L. lactis*.

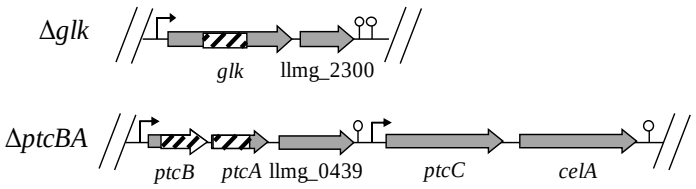

Fig. 3.1 – Schematic overview of the cellobiose-PTS operon and the glucokinase gene in the chromosome of *L. lactis* NZ9000 and its isogenic mutant strains. Hooked arrow, putative promoter; lollipop, putative terminator structure; dashed areas, deleted sequence (adapted from Castro, 2009).

The methodology used to introduce the point-mutations in the *ptn* genes generates a mixture of colonies with two possible genotypes (parental and mutated). To select the strains with the desired point-mutations we used the MAMA PCR technique (Cha *et al.*, 1992). This PCR-based method allows specific amplification of sequences differing by as little as a single base pair, by using a combination of three primers in the same PCR reaction where one (MAMA primer) is designed to contain at least one mismatch with a specific allele at the 3´end

(see Material and Methods section for details). In this work, the MAMA primer was designed to contain two mismatches with the wild-type allele and one mismatch with the mutated allele. Therefore, colonies carrying the native gene were expected to originate a single (big) band whereas one (small) or two fragments (small and big) were predicted in colonies with the mutated gene as a result of the MAMA PCR. Fig. 3.2 shows a typical DNA gel with the results of a MAMA PCR reaction in several colonies.

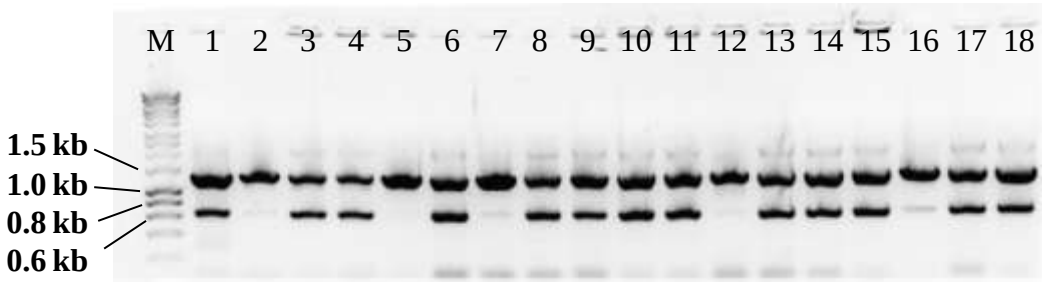

Fig. 3.2 - Agarose gel depicting DNA fragments obtained by Mismatch Amplification Mutation Assay (MAMA) PCR in *L. lactis* Δ*glk* (isolate 1) colonies resulting from the excision step to introduce *ptnC* point-mutation. If the native gene is present only one DNA fragment appears, in the case of a mutated gene two DNA fragments are amplified. M – DNA ladder; lines 1 to 18 – colonies 1 to 18.

The sequence of the mutated genes in the colonies selected by the MAMA PCR was further confirmed by sequencing analysis (Fig. 3.3). When compared to NZ9000, the sequence of *ptnC* in strains NZ9000*ptnC'* and Δ*glkptnC*' contains a single base change at nucleotide 331 (g to a) confirming the point-mutation at codon 111 (ggt::agt) that translates in a G111::S111 substitution. In the same way, the *ptnD* point-mutation at codon 105 was confirmed in strains NZ9000*ptnD'* and Δ*ptcBAptnD*', a single base modification at nucleotide 315 (g to a) originates the codon change atg::ata leading to M105::I105 substitution.

A

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NZ9000          1 atggaatacggtgttttatctgtaatcttggtcattctcgttgccttccttgctggtcttgaaggtatccttgaccaat
NZ9000ptnC'     1 atggaatacggtgttttatctgtaatcttggtcattctcgttgccttccttgctggtcttgaaggtatccttgaccaat
ΔglkptnC'       1 atggaatacggtgttttatctgtaatcttggtcattctcgttgccttccttgctggtcttgaaggtatccttgaccaat

               80 ggcaattccaccaaccaattatcgcctgctcgctcatcggtattgttactggtcacgcatctgcagggattatcctcgg
               80 ggcaattccaccaaccaattatcgcctgctcgctcatcggtattgttactggtcacgcatctgcagggattatcctcgg
               80 ggcaattccaccaaccaattatcgcctgctcgctcatcggtattgttactggtcacgcatctgcagggattatcctcgg

              159 tggttcgcttcaattgatcgctcttggttgggctaacgttggtgccgctgtcgcacccgatgctgcccttgcctcaatc
              159 tggttcgcttcaattgatcgctcttggttgggctaacgttggtgccgctgtcgcacccgatgctgcccttgcctcaatc
              159 tggttcgcttcaattgatcgctcttggttgggctaacgttggtgccgctgtcgcacccgatgctgcccttgcctcaatc

              238 gcctcatctattttgatggttcaatcaaacaactttgatttgactcacatcatgggtactatcgttcctgctgctatct
              238 gcctcatctattttgatggttcaatcaaacaactttgatttgactcacatcatgggtactatcgttcctgctgctatct
              238 gcctcatctattttgatggttcaatcaaacaactttgatttgactcacatcatgggtactatcgttcctgctgctatct

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              396 tgcggaaaatggttcttactcaggtgttgaaatgtggcacttcatcgcgcttatctgtcaaggtttgcgtatcgctatc
              396 tgcggaaaatggttcttactcaggtgttgaaatgtggcacttcatcgcgcttatctgtcaaggtttgcgtatcgctatc

              475 cctgctggacttcttttggttatctcacctgaagctatccaaaacgcacttgctgctattcctccagttatctctggtg
              475 cctgctggacttcttttggttatctcacctgaagctatccaaaacgcacttgctgctattcctccagttatctctggtg
              475 cctgctggacttcttttggttatctcacctgaagctatccaaaacgcacttgctgctattcctccagttatctctggtg

              554 gtcttgctgtcggtggtgggatggttgttgccgttggttatgcaatggttatcaaccttatggctactcgtgaagtatg
              554 gtcttgctgtcggtggtgggatggttgttgccgttggttatgcaatggttatcaaccttatggctactcgtgaagtatg
              554 gtcttgctgtcggtggtgggatggttgttgccgttggttatgcaatggttatcaaccttatggctactcgtgaagtatg

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              633 gcctttcttcttccttggtttcgctccgcacccaatctctgaattaacattgattgcaactggtgtcctcggtgttgtt

              712 atcgctatcgtttaccttaaccttcaatcttctggtggttctggaaatggtactgcatcatcatcaggtgacccaattg
              712 atcgctatcgtttaccttaaccttcaatcttctggtggttctggaaatggtactgcatcatcatcaggtgacccaattg
              712 atcgctatcgtttaccttaaccttcaatcttctggtggttctggaaatggtactgcatcatcatcaggtgacccaattg

              791 gcgacatcttgaacgactactaa
              791 gcgacatcttgaacgactactaa
              791 gcgacatcttgaacgactactaa
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B

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NZ9000          1 atgtctgataaaaaagtaactcttgataagaaaatccgtcgtagcgttatgtggcgttcaatgttcctccaaggttctt
NZ9000ptnD'     1 atgtctgataaaaaagtaactcttgataagaaaatccgtcgtagcgttatgtggcgttcaatgttcctccaaggttctt
ΔptcBAptnD'     1 atgtctgataaaaaagtaactcttgataagaaaatccgtcgtagcgttatgtggcgttcaatgttcctccaaggttctt

               80 ggaactacgaacgtatgcaaaatggtggttgggcttactcgctcattccagcattgaaaaaactctacccttctggcga
               80 ggaactacgaacgtatgcaaaatggtggttgggcttactcgctcattccagcattgaaaaaactctacccttctggcga
               80 ggaactacgaacgtatgcaaaatggtggttgggcttactcgctcattccagcattgaaaaaactctacccttctggcga

              159 agaagctaaagaagctttgaaacgtcacttggaattctttaatactcacccatacgttgctgctcctatcatcggtgta
              159 agaagctaaagaagctttgaaacgtcacttggaattctttaatactcacccatacgttgctgctcctatcatcggtgta
              159 agaagctaaagaagctttgaaacgtcacttggaattctttaatactcacccatacgttgctgctcctatcatcggtgta

              238 actcttgcccttgaagaagaacgtgctaacggtgctgatatcgatgatgccgctattcaaggggtaaaagttggtatga
              238 actcttgcccttgaagaagaacgtgctaacggtgctgatatcgatgatgccgctattcaaggggtaaaagttggtataa
              238 actcttgcccttgaagaagaacgtgctaacggtgctgatatcgatgatgccgctattcaaggggtaaaagttggtataa

              317 tgggtcctcttgccggtatcggtgacccagtcttctggtttacagttcgccctatcgttggtgctatcgcagcttcatt
              317 tgggtcctcttgccggtatcggtgacccagtcttctggtttacagttcgccctatcgttggtgctatcgcagcttcatt
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              712 gagattcttggtcaagtaggtaacaaactttctc
              712 gagattcttggtcaagtaggtaacaaactttctc
              712 gagattcttggtcaagtaggtaacaaactttctc
```

Fig. 3.3 – A - Alignment of *ptnC* gene sequence in the wild-type strain NZ9000 and mutated strains NZ9000*ptnC*' and Δ*glkptnC*'. B - Alignment of *ptnD* gene sequence in wild-type strain NZ9000 and mutated strains NZ9000*ptnD*' and Δ*ptcBAptnD*'. The single nucleotide changes are framed in red.

3. 2. Characterization of glucose metabolism by *ptnC* and *ptnD* mutant strains

3. 2. 1 Growth characteristics

The growth profiles of *ptnC'* and *ptnD'* mutant strains on glucose were compared to those of NZ9000 and its derivatives Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) (Fig. 3.4). All the strains were grown as batch cultures in CDM with 1% glucose (w/v) under anaerobic conditions and non-controlled pH (initial pH 6.5).

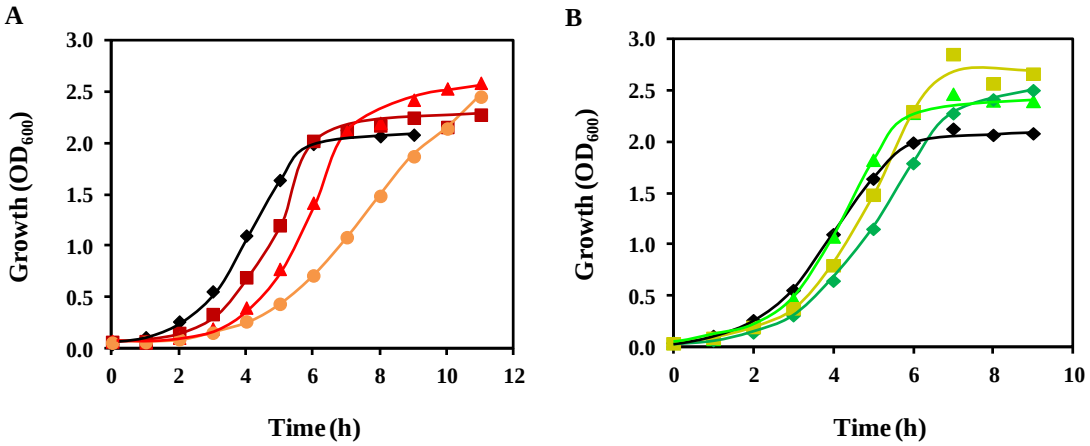

Fig. 3.4 – Growth profiles of *L. lactis* NZ9000 and the mutant strains in CDM with 1% glucose (w/v) at 30ºC and non-controlled pH (initial pH 6.5). (A) NZ9000, NZ9000*ptnC*', Δ*glk* (isolate 1), and Δ*glkptnC*'. (B) NZ9000, NZ9000*ptnD*', Δ*ptcBA* (isolate 1) and Δ*ptcBAptnD*'. Symbols: NZ9000 (◆), NZ9000*ptnC*' (▲), Δ*glk* (isolate 1) (■), Δ*glkptnC*' (●), NZ9000*ptnD*' (◆), Δ*ptcBA* (isolate 1) (▲) and Δ*ptcBAptnD*' (■). Lines are simple interpolations.

The differences observed between the growth profiles of NZ9000, Δ*glK* (isolate 1) and Δ*ptcBA* (isolate 1) were in accordance with previous profiles established during growth of these strains on glucose under controlled pH of 6.5 (Castro, 2009). It is evident that the specific point-mutations on *ptnC* and *ptnD* by itself affect negatively *L. lactis* growth, as denoted by the decrease in the specific growth rate of strains carrying the point-mutations (Table 3.1). Compared to the parental backgrounds NZ9000 and Δ*glk* (isolate 1), the *ptnC'* strains displayed a reduction of 19% (0.59 compared to 0.73 h^{-1}) and 33% (0.47 compared to 0.70 h^{-1}) in the specific growth rate, respectively. In contrast, the mutation on *ptnD* reduced the growth rate of NZ9000 by only 8% (0.67 compared to 0.73 h^{-1}) and 19% (0.65 compared to 0.81 h^{-1}) in the Δ*ptcBA* (isolate 1) strain.

Table 3.1 - Specific growth rates (μ) and generation time (t/n) of NZ9000 and mutant strains grown on glucose.

Strain	μ (h^{-1})	t/n (g)
NZ9000	0.73±0.05	0.96±0.08
Δ*glk* (isolate 1)	0.70±0.00	0.99±0.00
Δ*ptcBA* (isolate 1)	0.81±0.04	0.87±0.04
NZ9000*ptnC*'	0.59±0.01	1.18±0.03
NZ9000*ptnD*'	0.67±0.03	1.04±0.05
Δ*glkptnC*'	0.47±0.01	1.49±0.03
Δ*ptcBAptnD*'	0.65±0.00	1.07±0.00

Values of two independent experiments were averaged and are reported±standard deviation.

A comparison of the end-product yields derived from the metabolism of glucose during growth is presented in Table 3.2. Although lactate is the major product of glucose metabolism in all the strains studied, a slight shift to mixed-acid fermentation in NZ9000*ptnC'* and Δ*glKptnC'* mutants is clear. About 7% of the glucose consumed is converted to acetate, ethanol and formate. In strains NZ9000*ptnD'*, Δ*ptcBA* (isolate 1) and Δ*ptcBAptnD'* very small amounts of pyruvate were produced in addition to the other products. Altogether, these data indicate that the mutation on *ptnC* has a higher impact than that of *ptnD* on glucose metabolism of *L. lactis*.

Table 3.2 - Product yields from glucose metabolism in NZ9000 and glucose-transport mutant strains.

Strain	Product yield (mol product mol^{-1} glucose consumed)						
	Pyruvate	Lactate	Formate	Acetate	Ethanol	2,3-Butanediol	Acetoin
NZ9000	ND	1.75±0.14	ND	0.03±0.01	ND	0.01±0.01	0.01±0.01
NZ9000*ptnC*'	ND	1.39±0.12	0.28±0.04	0.15±0.03	0.15±0.00	<0.005	0.02±0.02
NZ9000*ptnD*'	<0.005	1.81±0.11	0.08±0.05	0.05±0.01	0.03±0.03	0.01±0.00	ND
Δ*glk* (isolate 1)	ND	1.71±0.21	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.00	0.01±0.01
Δ*ptcBA* (isolate 1)	<0.005	1.96±0.08	0.03±0.00	0.08±0.07	0.04±0.01	0.01±0.00	0.01±0.00
Δ*glkptnC*'	ND	1.34±0.16	0.24±0.00	0.14±0.01	0.17±0.01	0.01±0.00	<0.005
Δ*ptcBAptnD*'	0.01±0.00	1.94±0.01	0.04±0.01	0.07±0.04	0.03±0.01	0.01±0.00	0.01±0.00

Values of two independent experiments were averaged and are reported±standard deviation (SD).
ND – not detected.

3. 2. 2 Glycolytic dynamics

The effect of the specific amino acids changes on glucose metabolism was studied by *in vivo* ^{13}C-NMR in non-growing cell suspensions under an argon atmosphere and without pH control (buffer pH 6.5). Some of the advantages of using the *in vivo* NMR technique to study sugar metabolism in *L. lactis* have been illustrated by the information gathered in the laboratory during the past years (Neves *et al.*, 2005). These advantages include, besides allowing for non-invasive online measurements of intracellular metabolites, the detection of metabolic bottlenecks and new metabolic pathways, as well as the discrimination between the α- and β-anomers of the sugars (Neves *et al.*, 2000; Ramos *et al.*, 2004). The kinetics of glucose consumption and pools of intracellular metabolites in NZ9000 and mutant strains is depicted in Fig. 3.5. Strains Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) showed similar and slightly lower (1.5-fold), respectively, glucose consumption rates as compared to NZ9000. As expected from the results described above, both *ptnC* and *ptnD* mutations negatively affect substrate catabolism. Strains expressing *ptnC'* showed a reduction of about 50% in glucose consumption rate, while this parameter decreased 30 to 60% in *ptnD*' mutants. In general, the specific mutations on *ptnC* and *ptnD* clearly induce a preference for the consumption of β-glucose (Fig 3.5 and Table 3.3). A close analysis of the glucose consumption profiles in these strains revealed that substrate utilization is maximal while the β-anomeric form is available, slowing down after its exhaustion (Fig. 3.5).

In all strains, fructose 1,6-bisphosphate (FBP) reached the maximal concentration while glucose is available, starting to decrease when the β-glucose goes below 3mM. The maximal pool of FBP was only affected in strains Δ*glkptnC*' and Δ*ptcBAptnD*', decreasing from about 20 mM to 15 mM and 10 mM, respectively (Table 3.3). Strains NZ9000, NZ9000*ptnC'* and NZ9000*ptnD'* accumulated similar amounts (~10 mM) of 3-phosphoglycerate (3-PGA). However, the maximal concentration of this metabolite was about 5 mM in strains Δ*glk* (isolate 1), Δ*glkptnC'* and Δ*ptcBA* (isolate 1) and was below the detection limit in Δ*ptcBAptnD'* mutant. Interestingly, the mutations in the *ptnC* and *ptnD* of NZ9000 led to the accumulation of pyruvate, which reached maximal concentrations of 18 and 6 mM, respectively (Table 3.3). In both strains the pyruvate pool started to build-up during glucose consumption, however, while its maximal concentration remained fairly constant after substrate exhaustion in NZ9000*ptnD'* it decreased to undetectable levels in *NZ9000ptnC'* (Fig. 3.5). A similar profile of pyruvate accumulation to that of NZ9000*ptnC'* was observed in strain Δ*glKptnC'*. A transient pool of pyruvate (about 9 mM) was also observed in strain Δ*ptcBA* (isolate 1). In Δ*ptcBAptnD'*, pyruvate also accumulated to a maximal concentration of 9 mM at glucose onset, but it slowly decreased to about 6 mM by the end of the experiments.

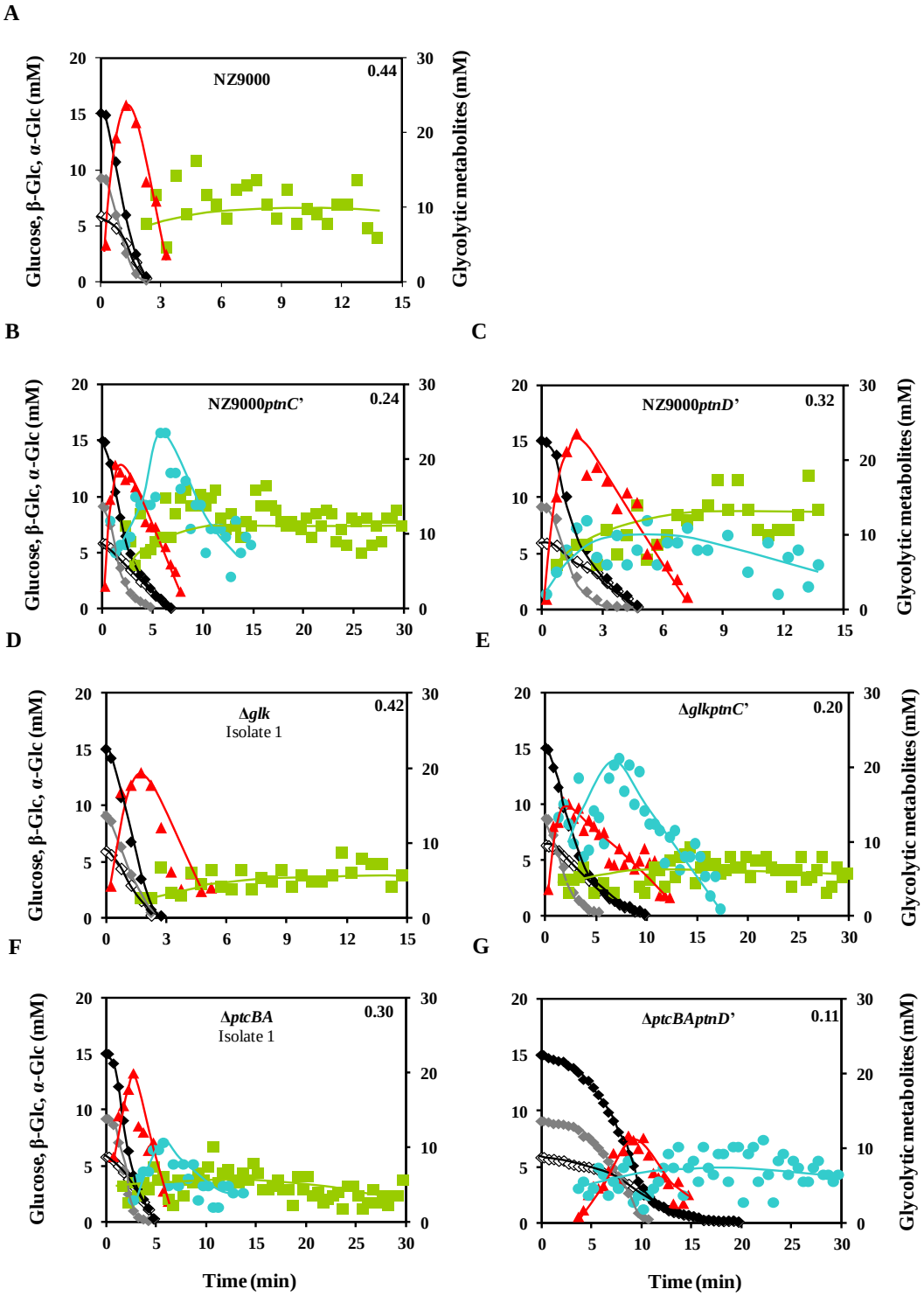

Fig. 3.5 – Kinetics of [1-^{13}C]glucose (15 mM) consumption and pools of intracellular metabolites in resting cells of *L. lactis* strains NZ9000 (A), NZ9000*ptnC*' (B), NZ9000*ptnD*' (C), Δ*glk* (isolate 1) (D), Δ*glkptnC*' (E), Δ*ptcBA* (isolate 1) (F), Δ*ptcBAptnD*' (G) under anaerobic conditions at 30ºC without pH control (initial pH 6.5). Maximal glucose consumption rates (GCR, µmol min^{-1} mg prot^{-1}) are indicated in upper-right corners. Symbols: total glucose (◆), β-glucose (◆), α-glucose (◇), fructose 1,6-biphosphate (▲), 3-phosphoglycerate (■), pyruvate (●). Graphics of a representative experiment.

Table 3.3 - Intracellular metabolites (mM) and glucose consumption rates (µmol min^{-1} mg $prot^{-1}$) from metabolism of 15 mM [1-^{13}C]glucose by *L. lactis* NZ9000 and mutant strains.

Strain	FBP	3-PGA	Pyruvate	GlcCR Total	GlcCR β-anomer	GlcCR α-anomer	β-/α-Glc
NZ9000[a]	22.4	9.2	ND	0.44	0.34	0.15	2.27
NZ9000*ptnC*'	17.6±1.6	9.1±6.7	18.0±4.0	0.23±0.01	0.19±0.02	0.05±0.01	3.75
NZ9000*ptnD*'	20.7±0.1	11.5±1.2	5.8±0.2	0.31±0.01	0.25±0.00	0.07±0.01	3.77
Δ*glk* (isolate 1)	16.3±3.1	4.7±1.2	ND	0.41±0.02	0.27±0.01	0.17±0.00	1.59
Δ*ptcBA* (isolate 1)	19.0±0.2	5.9±0.1	9.2±1.0	0.30±0.00	0.24±0.02	0.08±0.01	3.21
Δ*glkptnC*'	15.3±0.3	6.9±0.1	14.3±1.9	0.22±0.03	0.16±0.01	0.05±0.00	3.33
Δ*ptcBAptnD*'	9.8±2.3	ND	7.4±0.6	0.12±0.01	0.09±0.01	0.04±0.00	2.47

[a] – values of just one experiment.
Values of two independent experiments were averaged and are reported ± SD.
GlcCR – glucose consumption rate.
ND – not detected.

The pattern of end-products from glucose metabolism by the diverse strains was in line with that obtained during growth, with lactate being the major product. However, strains with mutation in *ptnC* showed a slight shift to mixed-acid fermentation, with a total of 10 to 13% of glucose being channeled for acetate, ethanol and 2,3-butanediol production. In addition, small amounts of aspartate (0.2-0.7 mM) and alanine (0.1-0.2 mM) were also detected in all strains (data not shown).

Table 3.4 – End-products (mM) obtained from the consumption of 15mM [1-^{13}C]glucose by *L. lactis* NZ9000 and mutant strains.

Strain	Lactate	Acetate	Ethanol	2,3-Butanediol
NZ9000[a]	26.3	0.22	ND	0.12
NZ9000*ptnC*'	22.2±3.3	1.0±0.1	1.0±0.1	0.4±0.0
NZ9000*ptnD*'	23.9±2.4	0.4±0.2	0.2±0.0	0.3±0.0
Δ*glk* (isolate 1)	25.0±0.3	ND	0.1±0.0	0.1±0.0
Δ*ptcBA* (isolate 1)	25.5±1.4	ND	0.1±0.0	0.1±0.0
Δ*glkptnC*'	22.0±1.0	1.3±0.3	1.8±0.1	0.5±0.1
Δ*ptcBAptnD*'	24.9±2.7	ND	0.3±0.2	0.2±0.0

[a] – values of just one experiment.
Values of two independent experiments were averaged and are reported ± standard deviation (SD).
ND – not detected.

3. 2. 3 Kinetic parameters of glucose uptake

The kinetic properties for glucose transport in whole cells of *L. lactis* NZ9000 and its derivatives were determined from [^{14}C]glucose uptake experiments using nonlinear least squares regression analysis to estimate V_{max} and K_m (Table 3.5).

Table 3.5 - Kinetic properties of glucose transport in whole cells of *L. lactis* NZ9000 and mutant strains.

Strain	V_{max}[b] (μmol min^{-1} mg prot^{-1})	K_m[b] (mM)	Transporters present
NZ9000[a]	0.20	21* 10^{-3}	PTS^{Man}, PTS^{Cel}, GlcU
NZ9000*ptnC*'	0.19±0.05	3.52±0.16	PTS^{Man}, PTS^{Cel}, GlcU
NZ9000*ptnD*'	0.15±0.02	1.38±0.04	PTS^{Man}, PTS^{Cel}, GlcU
Δ*glk* (isolate 1)	0.27±0.05	(22±4)*10^{-3}	PTS^{Man}, PTS^{Cel}, GlcU^{+}
Δ*ptcBA* (isolate 1)	0.17±0.02	(24±1)*10^{-3}	PTS^{Man}, GlcU
Δ*glkptnC*'	0.18±0.01 0.01±0.00	3.54±0.12 (48±15)*10^{-3}	PTS^{Man}, PTS^{Cel}, GlcU^{+}
Δ*ptcBAptnD*'	0.10[ac] 0.01±0.00	0.67[ac] (3±1)*10^{-3}	PTS^{Man}, GlcU

[a] - values obtained of one experiment.
[b] - values obtained of three independent experiments averaged and reported ± SD. V_{max} and K_m were determined using glucose concentrations varying as follows: NZ9000, 0.001 to 0.1 mM; NZ9000*ptnC*', 0.05 to 15 mM, NZ9000*ptnD*', 0.005 to 15 mM; Δ*glk* (isolate 1), 0.001 to 0.5 mM, Δ*ptcBA* (isolate 1), 0.001 to 5 mM, Δ*glkptnC*', 0.001 to 10 mM and Δ*ptcBAptnD*', 0.0005 to 5 mM.
[c] – transport determinations were performed in the presence of 2 mM of arginine.
[+] - The GlcU/Glk pathway for glucose dissimilation is blocked due to inactivation of *glk*.

Glucose transport in NZ9000 was characterized by high affinity (K_m, 21 μM) and capacity (V_{max}, 0.2 μmol min^{-1} mg prot^{-1}) and was in accordance with previous results reported by (Castro *et al.*, 2009). The kinetic properties determined for Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) were identical to those of NZ9000, indicating that transport is being conducted mainly by the PTS^{Man}. The affinity for glucose was considerably reduced in strains NZ9000*ptnC'* and NZ9000*ptnD'* reaching the mM range. Since in these strains the PTS^{Cel} and GlcU are fully functional, the low affinity for glucose indicates that the mutated amino acids in the permease domain of PTS^{Man} have a significant role in the sugar transport through this system. In strain Δ*glkptnC*' it was possible to distinguish two kinetics of Michaelis-Menten for glucose uptake. The first characterized by low affinity (K_m, 3.5 mM) and high capacity (V_{max}, 0.18 μmol min^{-1} mg prot^{-1}), typical of transport via the secondary transporter PTS^{Cel} (Castro *et al.*, 2009). In contrast, the second kinetic of glucose transport determined in Δ*glkptnC*' was characterized by high affinity (K_m, 48 μM) but low capacity (V_{max}, 0.01 μmol min^{-1} mg prot^{-1}). Taking into consideration the high affinity for glucose and that transport via the GlcU permease

is blocked in strain Δ*glkptnC*', this second kinetics must be the result of glucose uptake via the mutated PTS^{Man}. Strain Δ*ptcBAptnD'* had a high affinity for glucose (K_m, 3 μM), but its uptake capacity was also reduced (V_{max}, 0.01 μmol min^{-1} mg prot^{-1}); parameters similar to those determined for the mutated PTS^{Man} in Δ*glkptnC*'. In fact, glucose transport in strain Δ*ptcBAptnD'* can only occur via the mutated PTS^{Man} and GlcU. However, uptake via the permease is driven by the proton motive force requiring cell energization (Castro *et al.*, 2009). When the transport assay was performed in the presence of arginine, the affinity for glucose decreased about 220-fold and the V_{max} increased 10-fold, as compared to non-energized cells of Δ*ptcBAptnD'* (Table 3.5).

3. 3. Engineering succinate production in *L. lactis*

One of the metabolic consequences of sugar transport engineering, and in particular that of PTS systems, is an increased PEP/pyruvate potential (Gosset, 2005). In *L. lactis* glucose is mainly transported through PTS systems and it was shown above that strains with specific mutations in the EIICD (permease domain) of the PTS^{Man} were able to accumulate considerable amounts of pyruvate. This led us to surmise a beneficial role of the PTS mutations in the development of lactococcal hosts for the production of chemicals requiring a carboxylation step, such as succinate. To test this hypothesis, we attempted to overproduce the succinate biosynthetic routes in the mutants with the mutated PTS^{Man}, in particular the PEP and pyruvate carboxylases. Under anaerobic conditions, PEP or pyruvate can be carboxylated to OAA, by the action of specific carboxylases, which is further reduced to malate or aspartate and eventually converted to succinate.

The engineering strategy to investigate the effect of overproducing the PEP and pyruvate carboxylases in the mutant strains included the simultaneous overexpression of *pyc* and *ppc* genes of *Corynebacterium glutamicum* (Litsanov *et al.*, 2012b) under the control of the nisin inducible promoter P*nisA*. However, after three attempts and several controls we failed to clone the *pyc* gene in the expression vector pNZ8048. In over 150 colonies tested by PCR with primers specific for the *pyc* gene, just 5 colonies seemed to have the right construct which proved to be false positives after confirmation of the vector by enzymatic restriction (see Fig. 3.6 for an example).

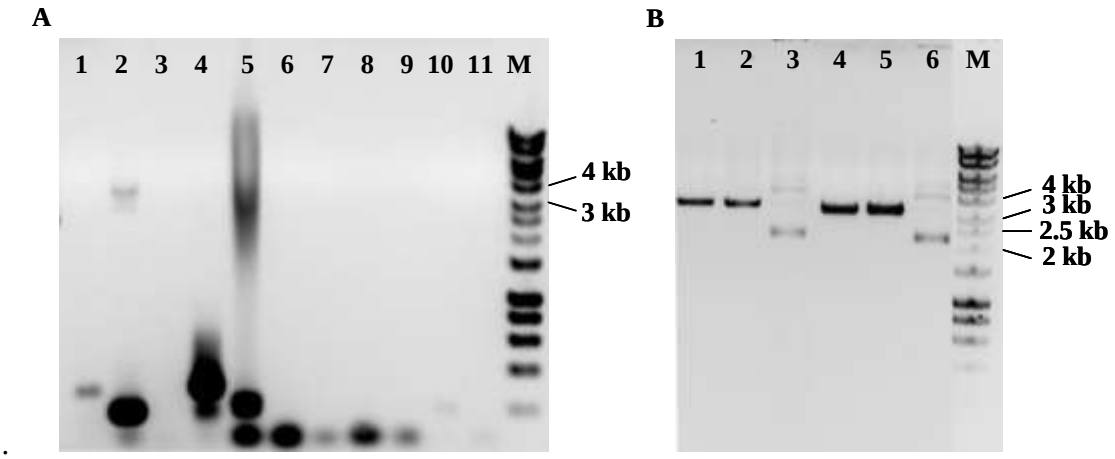

Fig. 3.6 – First attempt to construct a plasmid for *pyc* expression. (A) Selection of putative NZ9000 transformants with pNZ8048*pyc*$^{+}$. Each line shows the result of a colony PCR reaction with *pyc* specific primers. Expected band size: 3437 bp (B) Restriction pattern of plasmids purified from putative positive transformants digested with *Xba*I, *Bgl*II and *Eco*RI. Expected band sizes for pNZ8048*pyc*$^{+}$ with *Xba*I – 6759bp, *Bgl*II – 6515bp + 243bp and *Eco*RI – 4617bp + 1347bp + 795bp. Panel A: lines 1-10 – colonies 1-10, M – DNA ladder; Panel B: lines 1-3 – vector from colony 2 digested with *Xba*I, *Bgl*II and *Eco*RI, lines 4-6 - vector from colony 5 digested with *Xba*I, *Bgl*II and *Eco*RI, M – DNA ladder.

In a second approach to clone the *pyc* gene in pNZ8048, the restriction site included in the 5' end of the gene was changed from *Pci*I to *Bsp*HI (a restriction enzyme successfully used in the laboratory for cloning in pNZ8048) and the respective overhang extended to avoid any difficulties in the enzyme digestion. Despite all the changes made the cloning strategy failed again (Fig. 3.7).

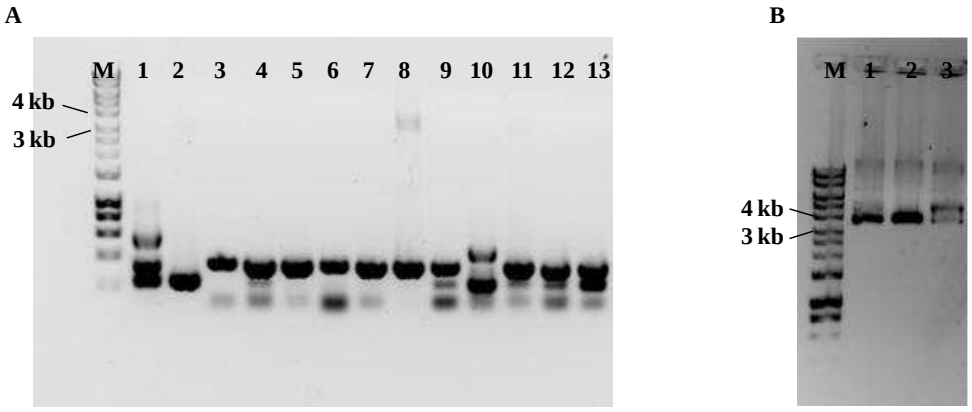

Fig. 3.7 - Second attempt to construct a plasmid for *pyc* expression. (A) Selection of putative NZ9000 transformants with pNZ8048*pyc*$^{+}$. Each line shows the result of a colony PCR reaction with *pyc* specific primers. Expected band size: 3437 bp (B) Restriction pattern of plasmid purified from putative positive transformants digested with *Sph*I, *Kpn*II and *Nde*I. Expected band sizes for pNZ8048*pyc*$^{+}$ with *Sph*I – 6759bp, KpnI – 3513bp + 3246bp and *Nde*I – 6759bp. Panel A: M – DNA ladder, lines 1- 13 – colonies 1-13; Panel B: M – DNA ladder, lines 1-3 – vector from colony 8 digested with *Sph*I, *Kpn*I and *Nde*I.

Given the unsuccessful attempts to clone the pyruvate carboxylase, we questioned if the high GC content of the coryneform gene would interfere with its cloning in *L. lactis*, a Gram-

positive organism with a low content in GC. Therefore, cloning of the *ppc* gene alone in pNZ8048 was attempted. In contrast to *pyc*, cloning of the *ppc* gene was easily achieved in the first attempt made (Fig. 3.8).

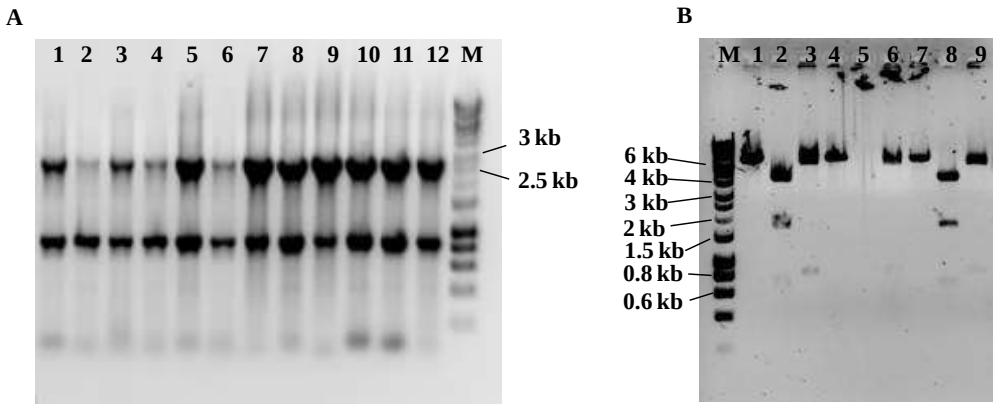

Fig 3. 8 - Construction of a plasmid for *ppc* expression. (A) Selection of putative NZ9000 transformants with pNZ8048*ppc*$^+$. Each line shows the result of a colony PCR reaction with *ppc* specific primers. Expected band size: 2798bp (B) Restriction pattern of plasmids purified from putative positive transformants digested with *XbaI*, *Xho*I and *Aat*II. Expected band sizes for pNZ8048*ppc*$^+$ with *Xba*I – 6117bp, *Xho*I – 3885bp + 1587bp + 645bp and *Aat*II – 5343bp + 774bp. Panel A: lines 1- 12 – colonies 1-12, M – DNA ladder; Panel B: M – DNA ladder, lines 1-3 – vector from colony 5 digested with *Xba*I, *Xho*I and *Aat*II, lines 4-6 – vector from colony 9 digested with *Xba*I, *Xho*I and *Aat*II, lines 7-9 – vector from colony 10 digested with *Xba*I, *Xho*I and *Aat*II.

One of the positive transformants NZ9000*ppc*$^+$ was selected for testing PEP carboxylase activity overexpression. NZ9000*ppc*$^+$ and the control strain NZ9000pNZ8048 were grown in complex media and expression induced by the addition of 1 μg/L nisin at OD_{600}=0.3 (Fig. 3.9). No PEP carboxylase activity was detected in extracts derived from the overproducing or the control strain using the enzyme assay described in Material and Methods section (data not shown). There are several factors which could limit expression and/or activity detection, but they were not investigated due to the deadline imposed to finish this Master thesis. These aspects will be further detailed in the Discussion section.

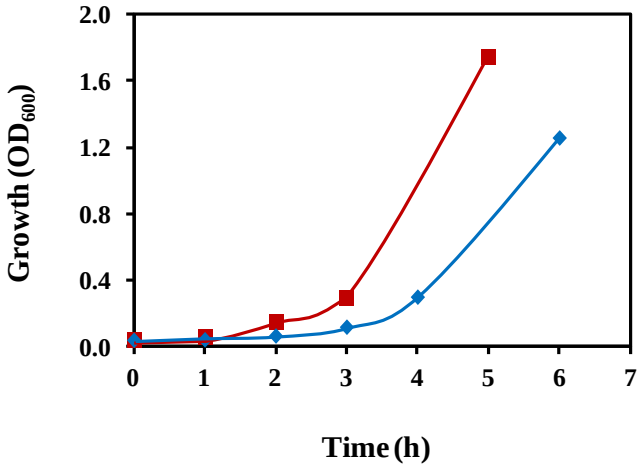

Fig 3.9 - Growth of *L. lactis* NZ9000pNZ8048 and *L. lactis* NZ9000*ppc*$^{+}$ in CDM with 1% glucose (w/v) at 30ºC. Chloramphenicol (5 mg/L) was added to the growth medium to maintain plasmid selection. Cells were induced with nisin 1 µg/L at OD_{600}= 0.3. Symbols: NZ9000pNZ8048 (■); NZ9000*ppc*$^{+}$ (◆).

4. Discussion

This work aimed to establish phenotype-genotype correlations in *L. lactis* NZ9000 (wild-type) and derived glucose transport mutants carrying specific point-mutations in PTS^{Man} permease, its main glucose transporter. Just by comparing the growth profiles of wild-type NZ9000 and mutated strains NZ9000*ptnC'* and NZ9000*ptnD'* it is immediately clear that the amino acid changes, introduced by site-directed mutagenesis, have only *per se* an impact in *L. lactis* physiology (Fig. 3.4). Both mutations reduce the growth rate of the wild-type strain. In addition, the NZ9000*ptnC'* strain also displayed a slight shift to a mixed-acid fermentation profile. Such effects point out towards a limitation at the level of the glycolytic flux which may result from defects on glucose transport (Castro *et al.*, 2009). The growth rates of Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1) were similar to that of NZ9000 and similar to preliminary determinations made in our laboratory (data not shown). Moreover, the values are in accordance with those previously reported for the same strains growing at pH 6.5 (Castro, 2009). Introduction of *ptnC* and *ptnD* point-mutations in Δ*glk* (isolate 1) and Δ*ptcBA* (isolate 1), respectively, also leads to a reduction of growth rate in both strains and to mild mixed-acid fermentation in Δ*glk* (isolate 1). Growth rate values of 0.51 (Δ*glK* isolate 2) and 0.55 h^{-1} (Δ*ptcBA* isolate 2) were determined for strains carrying the spontaneous point-mutations (Pool, 2008), and are in line with those now measured in Δ*glkptnC'* and Δ*ptcBAptnD'* (Table 3.1).

The effects of *ptnC* and *ptnD* mutations in NZ9000 are not restricted only to growth and fermentation profiles, they also caused a reduction in substrate consumption rates (48% in NZ9000*ptnC'* and 30% in NZ9000*ptnD'*) and an anomeric preference for the β-form of glucose, as determined by *in vivo* NMR (Fig. 3.5). This preference for β-glucose is typical of glucose consumption through PTS^{Cel} and/or GlcU (Castro *et al.*, 2009) and has been previously observed in Δ*glk* (isolate 2) strain (Fig. 1.8D). In fact, if profile and rate of glucose consumption of Δ*glk* (isolate 1), Δ*glk* (isolate 2) and Δ*glkptnC'* are compared it is obvious that Δ*glk* (isolate 1) metabolizes glucose in a way that resembles the wild-type strain NZ9000, whereas Δ*glk* (isolate 2) and Δ*glkptnC'* are similar between each other (compare Fig.1.8D and 3.5E). Moreover, the reduction observed in the glucose consumption rate in NZ9000*ptnC'* is similar to that caused by the introduction of *ptnC* point-mutation in Δ*glk* either spontaneously (47%) or by site-directed mutagenesis (46%). So, it can be postulated that the different metabolic behaviors formerly observed in both isolates of Δ*glk* strain are in fact due to the G111::S111 substitution in $EIIC^{Man}$ of isolate 2.

In contrast to the *ptnC* mutation, the site-directed change M105::I105 in $EIID^{Man}$ of Δ*ptcBA* (isolate 1) induced a glucose consumption profile distinct from that of Δ*ptcBA* (isolate

2) (compare Fig 1.8F and 3.5G). While glucose consumption rate was considerable reduced in Δ*ptcBA* (isolate 2), about 55% when compared to that of Δ*ptcBA* (isolate 1), no preference for any of the anomeric forms of glucose was observed. However, the Δ*ptcBAptnD'* shows a marked preference for β-glucose and a kinetics of sugar consumption rather complex, an initial lag-phase is followed by a period of acceleration, with the rate varying up to a maximum of 0.12 μmol min^{-1} mg $prot^{-1}$. This latter pattern is typical of *L. lactis* strains where the glucose uptake is restricted to GlcU (Castro *et al.*, 2009). Although this discrepancy could not be immediately explained, the preference for β-glucose in Δ*ptcBAptnD'* was in agreement with the same preference induced by the *ptnD* mutation in the wild-type strain NZ9000. In addition, the Δ*ptcBA* (isolate 1) strain also displayed the same anomeric preference during the metabolism of 15 mM glucose under non-controlled conditions of pH (initial pH 6.5), an observation that was in total disagreement with the previous characterization made when 20 mM of glucose was consumed at a constant pH of 6.5. Until this point, all the data gathered for Δ*ptcBA* (isolate 1) indicated that its metabolic behavior was identical to the wild-type strain, reason why a spontaneous point-mutation identified in the *ptnAB* gene (encoding the cytoplasmatic $EIIAB^{Man}$ domain) of this strain was disregarded and considered to have no effect on glucose transport of *L. lactis*. The point-mutation in the *ptnAB* causes the change of aspartate 237 to alanine in $EIIAB^{Man}$, and results from a single base substitution (a::c) at position 710 bp of the gene. It is possible that the effects caused by this mutation in *ptnAB* gene are just detectable under non-controlled conditions of pH and/or glucose concentrations ≤ 15 mM. This would imply that the metabolic profile of Δ*ptcBAptnD'* is the result from the cumulative effect of two mutations in the PTS^{Man} and PTS^{Cel} inactivation, making glucose uptake through GlcU more notorious. Indeed, it was shown that non-PTS permease(s) in *Streptococcus mutans* become functional at low pH (Cvitkovitch *et al.*, 1995; Hamilton and Ellwood, 1978). Furthermore, it has been described that an acidic environment has a negative effect on PTS activity (Vadeboncoeur *et al.*, 1991). If all these putative explanations are true it also means that the anomeric preference induced by *ptnD*, but not *ptnC*, mutation would be sensitive to a non-controlled pH and/or glucose concentrations ≤ 15 mM. This hypothesis is based on the fact that Δ*ptcBA* (isolate 2) with the spontaneous point-mutation on *ptnD* does not have preference for any of the glucose anomers. Confirmation of the hypothesis suggested above would require a characterization of *ptnD* mutants under controlled conditions of pH (6.5) and/or glucose concentrations above 15mM. Additionally, change of the mutated amino acid by the native aspartate 237 in $EIIAB^{Man}$ of strain Δ*ptcBAptnD'* would also be important.

In general, the kinetic parameters of glucose transport determined in whole cells of the diverse mutants are in accordance with the previous results obtained for strains carrying the spontaneous $EIICD^{Man}$ mutations (compare Table 1.5 and 3.5). The only exception is the

maximal capacity of glucose uptake measured in non-energized cells of Δ*ptcBAptnD'* (0.01 µmol min^{-1} mg $prot^{-1}$), which is 9-fold lower than that calculated for Δ*ptcBA* (isolate 2). Such a difference is probably related with the double point-mutation in the PTS^{Man} of Δ*ptcBAptnD'* strain, as explained above. Nevertheless, it was possible to demonstrate that the mutated amino acids in the permease domain of PTS^{Man} have a major impact in the sugar transport through this system. The data obtained indicates that both *ptnC* and *ptnD* point-mutations negatively affect the capacity but not the affinity of PTS^{Man}. An effect that is only noticeable in double mutant strains Δ*glkptnC'* and Δ*ptcBAptnD'*. If the mutations have any impact on the anomeric preference of PTS^{Man} remains elusive. An answer to this question requires that the point-mutations would be introduced in a strain with only the PTS^{Man} operative.

Based on the different physiological profiles is tempting to say that the *ptnC* mutation is more deleterious than that of *ptnD*. Both $EIIC^{Man}$ and $EIID^{Man}$ are necessary for glucose transport, but the IIC^{Man} presumably comprises all or most of the glucose transporting channels (Huber and Erni, 1996). Therefore, is not surprising that a change in *ptnC* could have a major impact in the overall structure of PTS^{Man} permease. The molecular mechanisms behind the observed effects caused by not only the mutations herein indentified, but also by other possible mutations are hampered by the lack of PTS permease structures. Nevertheless, this gap may be filled-in in a near future as testified by the recent appearance in the literature of reports unraveling the structure of the PTS^{Glc} permease ($EIIC^{Glc}$) from *Escherichia coli* (Jeckelmann *et al.*, 2011; Zurbriggen *et al.*, 2010). It is important to refer that the Gly111 is highly conserved among sequences of $EIIC^{Man}$ and the Met105 of $EIID^{Man}$ is present in all *Firmicutes*.

The 3-PGA levels of parental and mutated strains were not significantly different, except in strains Δ*ptcBA* and Δ*ptcBAptnD'* (Table 3.3). Nevertheless, pyruvate accumulation was a constant feature in all the strains carrying the point-mutations indicating an increased PEP/pyruvate potential. As a way to test the usefulness of glucose transport mutants as platforms for succinate production, attempts were made to overproduce the *pyc* and *ppc* carboxylases of *Corynebacterium glutamicum* in strains with *ptnC* and *ptnD* mutations. The carboxylase genes were chosen based on the high succinate yields obtained when overproduced in the native organism (Litsanov *et al.*, 2012b). Despite all the efforts reported in the Results section, we failed to clone the *pyc* gene in the lactococcal expression vector pNZ8048. Although *L. lactis* is quite amenable to the expression of heterologous genes with high GC content, it can happen that in this particular case the coryneform gene may be toxic to the cells. An alternative could be the expression of a synthetic *pyc* gene with optimized codon usage for *L. lactis*. In addition, overproduction of the native lactococcal pyc, encoded by the *pycA* gene, can be considered. This gene has already been used in combination with the PEP carboxylase from *Sorghum vulgare* to improve succinate production in *E. coli* (Lin *et al.*, 2005c).

In contrast to *pyc* gene, cloning of *ppc* in the expression vector was relatively easy. However, ppc activity was not detected in cell extracts of NZ9000*ppc*$^{+}$. Factors such as errors in the gene sequence, confirmation and optimization of expression conditions can contribute for this null result and should be evaluated before proceeding with an alternative strategy. It is also possible that extra activators are missing in the enzymatic assay used. Besides DTT, acetyl-CoA and dioxane have been used as activators of PPC (Hasegawa *et al.*, 2008; Teraoka *et al.*, 1974). As a final resource the use of a synthetic gene can be considered.

5. Conclusion

The work of this thesis addresses the metabolic effect of specific point-mutations in the main *L. lactis* glucose transporter, the PTS^{Man}. Two spontaneous amino acid changes previously identified in the permease domain $EIICD^{Man}$ where introduced in *L. lactis* NZ9000 (wild-type) and glucose transport mutant derivatives by site-directed mutagenesis. A detailed characterization of growth, product formation, intracellular metabolite pools, glucose consumption and anomeric preference, as well as determination of kinetic parameters of glucose transport in whole cells was performed for the distinct strains.

The changes G111::S111 at $EIIC^{Man}$ and M105::I105 at $EIID^{Man}$ have an overall negative impact in growth and glucose consumption rates as well as in glucose transport affinity of *L. lactis*. Such effects are even more drastic when the point-mutations are combined with the inactivation of alternative glucose transporters (PTS^{Cel} and GlcU). In fact, it was possible to correlate the metabolic profiles of the spontaneous PTS^{Man} mutants with its specific amino acid changes, at least for *ptnC* point-mutation. The correlation is not so clear for the *ptnD* point-mutation in the Δ*ptcBA*, most probably because of second mutation in the PTS^{Man} cytoplasmatic domain ($EIIBA^{Man}$) of this strain. Since all the Δ*ptcBA* characterization available so far indicated that its PTS^{Man} was fully functional, this second point-mutation identified in $EIIAB^{Man}$ had been considered silent until now. A real understanding of the *ptnD* mutation effects on glucose transport and consumption profiles required further strain manipulation and/or specific experimental conditions, which were impossible to achieve within the time frame of this work.

The results obtained indicate that both point-mutations cause a decrease in the capacity of PTS^{Man} to transport glucose whereas the affinity for the sugar is maintained. The PTS^{Man} takes up both β- and α-anomers of glucose equally (Castro *et al.*, 2009). It should be interesting to determine if the point-mutations in the permease domain induce any changes in this anomeric preference. This feature can be easily assessed by introducing the specific amino acid changes in strain NZ9000Δ*ptcC*Δ*glcU*, which transports glucose only by the PTS^{Man} (Castro *et al.*, 2009).

The negative effects on growth and glucose consumption rates as well as differences in product and metabolite the profiles caused by $EIIC^{Man}$ mutation are bigger than those of $EIID^{Man}$, which was tentatively explained by the different number of glucose transporting channels that each protein comprises. The complete structure of PTS^{Man} or at least of its permease is of chief importance to fully understand glucose transport through this system and would assist in unravel the molecular mechanisms underlying the differences observed.

Although the evaluation of PTS^{Man} mutants for succinate production was not accomplished, the high levels of pyruvate accumulated by these strains strengths its potential as

hosts to engineer the production of value-added compounds that use this metabolite as precursor. Interestingly, preliminary results on modeling the dynamics of *L. lactis* central metabolism for strain development conducted by our collaborators from the INESC team, indicate a modest positive effect of PTS^{Man} in the production of 2,3-butanediol, an important chemical feed-stock with a wide range of applications (Costa *et al.*, 2012). In fact, the PTS^{Man} mutants characterized in this work showed a modest increase in the 2,3-butanediol levels as compared to their parent strains (Table 3.4).

In conclusion, this work discloses two key amino acids in the functionality of the main glucose transporter in *L. lactis*. Taking into consideration that they are highly conserved among the $EIICD^{Man}$ of other bacteria, it is expected that mutations herein identified would have a similar impact on glucose transport and metabolism in those organisms. Moreover, they modulate carbon fluxes without the need to knock-out complete domains of PTS transporters, which is expected to open the way to a wide range of applications.

6. References

Andersson, U., Levander, F., and Radstrom, P. (2001). Trehalose-6-phosphate phosphorylase is part of a novel metabolic pathway for trehalose utilization in *Lactococcus lactis*. *J Biol Chem* **276**, 42707-42713.

Andersson, U., and Radstrom, P. (2002). Physiological function of the maltose operon regulator, MalR, in *Lactococcus lactis*. *BMC Microbiol* **2**, 28.

Barabote, R.D., and Saier, M.H., Jr. (2005). Comparative genomic analyses of the bacterial phosphotransferase system. *Microbiol Mol Biol Rev* **69**, 608-634.

Barriere, C., Veiga-da-Cunha, M., Pons, N., Guedon, E., van Hijum, S.A., Kok, J., Kuipers, O.P., Ehrlich, D.S., and Renault, P. (2005). Fructose utilization in *Lactococcus lactis* as a model for low-GC gram-positive bacteria: its regulator, signal, and DNA-binding site. *J Bacteriol* **187**, 3752-3761.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293-300.

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**, 243-255.

Boels, I.C., Van Kranenburg, R., Kanning, M.W., Chong, B.F., De Vos, W.M., and Kleerebezem, M. (2003). Increased exopolysaccharide production in *Lactococcus lactis* due to increased levels of expression of the NIZO B40 eps gene cluster. *Appl Environ Microbiol* **69**, 5029-5031.

Bolotin, A., Quinquis, B., Ehrlich, S.D., and Sorokin, A. (2012). Complete genome sequence of *Lactococcus lactis* subsp. *cremoris* A76. *J Bacteriol* **194**, 1241-1242.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D., and Sorokin, A. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* **11**, 731-753.

Burgess, C., O'Connell-Motherway, M., Sybesma, W., Hugenholtz, J., and van Sinderen, D. (2004). Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods. *Appl Environ Microbiol* **70**, 5769-5777.

Cabrera-Valladares, N., Martinez, L.M., Flores, N., Hernandez-Chavez, G., Martinez, A., Bolivar, F., and Gosset, G. (2012). Physiologic Consequences of Glucose Transport and Phospho*enol*pyruvate Node Modifications in *Bacillus subtilis* 168. *J Mol Microbiol Biotechnol* **22**, 177-197.

Carvalho, A.L., Cardoso, F.S., Bohn, A., Neves, A.R., and Santos, H. (2011). Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance. *Appl Environ Microbiol* **77**, 4189-4199.

Castro, R. (2009). Sugar Metabolism in *Lactococcus lactis*. Glucose Transporters and a Novel α-Phosphoglucomutase. Ph.D. thesis, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Lisboa.

Castro, R., Neves, A.R., Fonseca, L.L., Pool, W.A., Kok, J., Kuipers, O.P., and Santos, H. (2009). Characterization of the individual glucose uptake systems of *Lactococcus lactis*: mannose-PTS, cellobiose-PTS and the novel GlcU permease. *Mol Microbiol* **71**, 795-806.

Cha, R.S., Zarbl, H., Keohavong, P., and Thilly, W.G. (1992). Mismatch amplification mutation assay (MAMA): application to the *c-H-ras* gene. *PCR Methods Appl* **2**, 14-20.

Chien, L.J., and Lee, C.K. (2007). Hyaluronic acid production by recombinant *Lactococcus lactis*. *Appl Microbiol Biotechnol* **77**, 339-346.

Citti, J.E., Sandine, W.E., and Elliker, P.R. (1967). Lactose and maltose uptake by *Streptococcus lactis*. *J Dairy Sci* **50**, 485-487.

Cocaign-Bousquet, M., Even, S., Lindley, N.D., and Loubiere, P. (2002). Anaerobic sugar catabolism in *Lactococcus lactis*: genetic regulation and enzyme control over pathway flux. *Appl Microbiol Biotechnol* **60**, 24-32.

Costa, R.S., Hartmann, A., Gaspar, P., Santos, H., Neves, A.R., and Vinga, S. (2012). Modeling the dynamics of *Lactococcus lactis* central metabolism for strain development. In 11th European Conference in Computational Biology.

Cox, S.J., Shalel Levanon, S., Sanchez, A., Lin, H., Peercy, B., Bennett, G.N., and San, K.Y. (2006). Development of a metabolic network design and optimization framework incorporating implementation constraints: a succinate production case study. *Metab Eng* **8**, 46-57.

Cvitkovitch, D.G., Boyd, D.A., Thevenot, T., and Hamilton, I.R. (1995). Glucose transport by a mutant of *Streptococcus mutans* unable to accumulate sugars via the phospho*enol*pyruvate phosphotransferase system. *J Bacteriol* **177**, 2251-2258.

D'Souza, R., Pandeya, D.R., Rahman, M., Seo Lee, H., Jung, J.K., and Hong, S.T. (2012). Genetic engineering of *Lactococcus lactis* to produce an amylase inhibitor for development of an anti-diabetes biodrug. *New Microbiol* **35**, 35-42.

Davidson, A.L., and Chen, J. (2004). ATP-binding cassette transporters in bacteria. *Annu Rev Biochem* **73**, 241-268.

de Ruyter, P.G., Kuipers, O.P., and de Vos, W.M. (1996). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62**, 3662-3667.

de Vos, W.M. (1987). Gene cloning and expression in lactic streptococci. *FEMS Microbiology Letters* **46**, 281-295.

de Vos, W.M., Boerrigter, I., van Rooyen, R.J., Reiche, B., and Hengstenberg, W. (1990). Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. *J Biol Chem* **265**, 22554-22560.

de Vos, W.M., and Hugenholtz, J. (2004). Engineering metabolic highways in Lactococci and other lactic acid bacteria. *Trends Biotechnol* **22**, 72-79.

de Vos, W.M., Kuipers, O.P., van der Meer, J.R., and Siezen, R.J. (1995). Maturation pathway of nisin and other antibiotics: post-translationally modified antimicrobial peptides exported by gram-positive bacteria. *Mol Microbiol* **17**, 427-437.

Ehrmann, M., Ehrle, R., Hofmann, E., Boos, W., and Schlosser, A. (1998). The ABC maltose transporter. *Mol Microbiol* **29**, 685-694.

Eikmanns, B.J., Follettie, M.T., Griot, M.U., and Sinskey, A.J. (1989). The phospho*enol*pyruvate carboxylase gene of *Corynebacterium glutamicum*: molecular cloning, nucleotide sequence, and expression. *Mol Gen Genet* **218**, 330-339.

Escalante, A., Salinas Cervantes, A., Gosset, G., and Bolivar, F. (2012). Current knowledge of the *Escherichia coli* phospho*enol*pyruvate-carbohydrate phosphotransferase system: peculiarities of regulation and impact on growth and product formation. *Appl Microbiol Biotechnol* **94**, 1483-1494.

Foucaud, C., and Poolman, B. (1992). Lactose transport system of *Streptococcus thermophilus*. Functional reconstitution of the protein and characterization of the kinetic mechanism of transport. *J Biol Chem* **267**, 22087-22094.

Gao, Y., Lu, Y., Teng, K.L., Chen, M.L., Zheng, H.J., Zhu, Y.Q., and Zhong, J. (2011). Complete genome sequence of *Lactococcus lactis* subsp. *lactis* CV56, a probiotic strain isolated from the vaginas of healthy women. *J Bacteriol* **193**, 2886-2887.

Gaspar, P., Neves, A.R., Gasson, M.J., Shearman, C.A., and Santos, H. (2011). High yields of 2,3-butanediol and mannitol in *Lactococcus lactis* through engineering of NAD(+) cofactor recycling. *Appl Environ Microbiol* **77**, 6826-6835.

Gaspar, P., Neves, A.R., Ramos, A., Gasson, M.J., Shearman, C.A., and Santos, H. (2004). Engineering *Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system. *Appl Environ Microbiol* **70**, 1466-1474.

Gaspar, P., Neves, A.R., Santos, H., Palencia, P.F., Peláez, C., and Requena, T. (2008). Engineering and re-routing of the metabolism of lactic acid bacteria. Mayo,B, López,P & Pérez-Martínez,G (Eds) Molecular Aspects of Lactic Acid Bacteria for Tradicional and New Applicaions Research Signpost, Fort PO, Kerala, India, 265-289.

Gasson, M.J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**, 1-9.

Gorke, B., and Stulke, J. (2008). Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**, 613-624.

Gosset, G. (2005). Improvement of *Escherichia coli* production strains by modification of the phospho*enol*pyruvate:sugar phosphotransferase system. *Microb Cell Fact* **4**, 14.

Grossiord, B.P., Luesink, E.J., Vaughan, E.E., Arnaud, A., and de Vos, W.M. (2003). Characterization, expression, and mutation of the *Lactococcus lactis galPMKTE* genes, involved in galactose utilization via the Leloir pathway. *J Bacteriol* **185**, 870-878.

Hamilton, I.R., and Ellwood, D.C. (1978). Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various pH values in a chemostat. *Infect Immun* **19**, 434-442.

Hasegawa, T., Hashimoto, K., Kawasaki, H., and Nakamatsu, T. (2008). Changes in enzyme activities at the pyruvate node in glutamate-overproducing *Corynebacterium glutamicum*. *J Biosci Bioeng* **105**, 12-19.

Hermann, B.G., Blok, K., and Patel, M.K. (2007). Producing bio-based bulk chemicals using industrial biotechnology saves energy and combats climate change. *Environ Sci Technol* **41**, 7915-7921.

Hernandez, I., Molenaar, D., Beekwilder, J., Bouwmeester, H., and van Hylckama Vlieg, J.E. (2007). Expression of plant flavor genes in *Lactococcus lactis*. *Appl Environ Microbiol* **73**, 1544-1552.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-59.

Holo, H., and Nes, I.F. (1995). Transformation of *Lactococcus* by electroporation. *Methods Mol Biol* **47**, 195-199.

Hols, P., Kleerebezem, M., Schanck, A.N., Ferain, T., Hugenholtz, J., Delcour, J., and de Vos, W.M. (1999). Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. *Nat Biotechnol* **17**, 588-592.

Huber, F., and Erni, B. (1996). Membrane topology of the mannose transporter of *Escherichia coli* K12. *Eur J Biochem* **239**, 810-817.

Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., de Vos, W., and Hols, P. (2000). *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Appl Environ Microbiol* **66**, 4112-4114.

Jeckelmann, J.M., Harder, D., Mari, S.A., Meury, M., Ucurum, Z., Muller, D.J., Erni, B., and Fotiadis, D. (2011). Structure and function of the glucose PTS transporter from *Escherichia coli*. *J Struct Biol* **176**, 395-403.

Jensen, N.B., Melchiorsen, C.R., Jokumsen, K.V., and Villadsen, J. (2001). Metabolic behavior of *Lactococcus lactis* MG1363 in microaerobic continuous cultivation at a low dilution rate. *Appl Environ Microbiol* **67**, 2677-2682.

Jensen, P.R., and Hammer, K. (1993). Minimal Requirements for Exponential Growth of *Lactococcus lactis*. *Appl Environ Microbiol* **59**, 4363-4366.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* **49**, 209-224.

Khalid, K. (2011). An overview of lactic acid bacteria. *Int. J. of Biosciences* **1**, 1-13.

Kleerebezem, M., and Hugenholtz, J. (2003). Metabolic pathway engineering in lactic acid bacteria. *Curr Opin Biotechnol* **14**, 232-237.

Konings, W.N., Kok, J., Kuipers, O.P., and Poolman, B. (2000). Lactic acid bacteria: the bugs of the new millennium. *Curr Opin Microbiol* **3**, 276-282.

Kowalczyk, M., Cocaign-Bousquet, M., Loubiere, P., and Bardowski, J. (2008). Identification and functional characterisation of cellobiose and lactose transport systems in *Lactococcus lactis* IL1403. *Arch Microbiol* **189**, 187-196.

Kuipers, O.P., De Ruyter, P.G.G.A., Kleerebezem, M., and de Vos, W. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol* **64**, 15-21.

Kundig, W., Ghosh, S., and Roseman, S. (1964). Phosphate Bound to Histidine in a Protein as an Intermediate in a Novel Phospho-Transferase System. *Proc Natl Acad Sci U S A* **52**, 1067-1074.

Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G., and Leenhouts, K. (1995). A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol* **177**, 7011-7018.

Lee, S.J., Bohm, A., Krug, M., and Boos, W. (2007). The ABC of binding-protein-dependent transport in Archaea. *Trends Microbiol* **15**, 389-397.

Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G., and Kok, J. (1996). A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* **253**, 217-224.

Levering, J., Musters, M.W., Bekker, M., Bellomo, D., Fiedler, T., de Vos, W.M., Hugenholtz, J., Kreikemeyer, B., Kummer, U., and Teusink, B. (2012). Role of phosphate in the central metabolism of two lactic acid bacteria--a comparative systems biology approach. *FEBS J* **279**, 1274-1290.

Lin, H., Bennett, G.N., and San, K.Y. (2005a). Chemostat culture characterization of *Escherichia coli* mutant strains metabolically engineered for aerobic succinate production: a study of the modified metabolic network based on metabolite profile, enzyme activity, and gene expression profile. *Metab Eng* **7**, 337-352.

Lin, H., Bennett, G.N., and San, K.Y. (2005b). Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. *Metab Eng* **7**, 116-127.

Lin, H., San, K.Y., and Bennett, G.N. (2005c). Effect of *Sorghum vulgare* phospho*enol*pyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. *Appl Microbiol Biotechnol* **67**, 515-523.

Linares, D.M., Kok, J., and Poolman, B. (2010). Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. *J Bacteriol* **192**, 5806-5812.

Litsanov, B., Brocker, M., and Bott, M. (2012a). Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. *Appl Environ Microbiol* **78**, 3325-3337.

Litsanov, B., Kabus, A., Brocker, M., and Bott, M. (2012b). Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. *Microb Biotechnol* **5**, 116-128.

Liu, J.Y., Li, A.H., Ji, C., and Yang, W.M. (2009). First description of a novel Weissella species as an opportunistic pathogen for rainbow trout *Oncorhynchus mykiss* (Walbaum) in China. *Vet Microbiol* **136**, 314-320.

Liu, S., Dien, B.S., and Cotta, M.A. (2005). Functional expression of bacterial *Zymobacter palmae* pyruvate decarboxylase gene in *Lactococcus lactis*. *Curr Microbiol* **50**, 324-328.

Lolkema, J.S., Poolman, B., and Konings, W.N. (1998). Bacterial solute uptake and efflux systems. *Curr Opin Microbiol* **1**, 248-253.

Lorca, G.L., Barabote, R.D., Zlotopolski, V., Tran, C., Winnen, B., Hvorup, R.N., Stonestrom, A.J., Nguyen, E., Huang, L.W., Kim, D.S., *et al.* (2007). Transport capabilities of eleven gram-positive bacteria: comparative genomic analyses. *Biochim Biophys Acta* **1768**, 1342-1366.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., *et al.* (2006). Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sci U S A* **103**, 15611-15616.

McKay, L.L., Walter, L.A., Sandine, W.E., and Elliker, P.R. (1969). Involvement of phospho*enol*pyruvate in lactose utilization by group N streptococci. *J Bacteriol* **99**, 603-610.

Melchiorsen, C.R., Jensen, N.B., Christensen, B., Vaever Jokumsen, K., and Villadsen, J. (2001). Dynamics of pyruvate metabolism in *Lactococcus lactis*. *Biotechnol Bioeng* **74**, 271-279.

Morello, E., Bermudez-Humaran, L.G., Llull, D., Sole, V., Miraglio, N., Langella, P., and Poquet, I. (2008). *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *J Mol Microbiol Biotechnol* **14**, 48-58.

Neves, A.R., Pool, W.A., Kok, J., Kuipers, O.P., and Santos, H. (2005). Overview on sugar metabolism and its control in *Lactococcus lactis* - the input from in vivo NMR. *FEMS Microbiol* Rev **29**, 531-554.

Neves, A.R., Pool, W.A., Solopova, A., Kok, J., Santos, H., and Kuipers, O.P. (2010). Towards enhanced galactose utilization by *Lactococcus lactis*. *Appl Environ Microbiol* **76**, 7048-7060.

Neves, A.R., Ramos, A., Nunes, M.C., Kleerebezem, M., Hugenholtz, J., de Vos, W.M., Almeida, J., and Santos, H. (1999). In vivo nuclear magnetic resonance studies of glycolytic kinetics in *Lactococcus lactis*. *Biotechnol Bioeng* **64**, 200-212.

Neves, A.R., Ramos, A., Shearman, C., Gasson, M.J., Almeida, J.S., and Santos, H. (2000). Metabolic characterization of *Lactococcus lactis* deficient in lactate dehydrogenase using in vivo 13C-NMR. *Eur J Biochem* **267**, 3859-3868.

Neves, A.R., Ventura, R., Mansour, N., Shearman, C., Gasson, M.J., Maycock, C., Ramos, A., and Santos, H. (2002). Is the glycolytic flux in *Lactococcus lactis* primarily controlled by the redox charge? Kinetics of NAD(+) and NADH pools determined in vivo by 13C NMR. *J Biol Chem* **277**, 28088-28098.

Papagianni, M. (2012). Recent advances in engineering the central carbon metabolism of industrially important bacteria. *Microb Cell Fact* **11**, 50.

Pfeiler, E.A., and Klaenhammer, T.R. (2007). The genomics of lactic acid bacteria. *Trends Microbiol* **15,** 546-553.

Pinto, J.P., Kuipers, O.P., Marreddy, R.K., Poolman, B., and Kok, J. (2011a). Efficient overproduction of membrane proteins in *Lactococcus lactis* requires the cell envelope stress sensor/regulator couple CesSR. *PLoS One* **6**, e21873.

Pinto, J.P., Zeyniyev, A., Karsens, H., Trip, H., Lolkema, J.S., Kuipers, O.P., and Kok, J. (2011b). pSEUDO, a genetic integration standard for *Lactococcus lactis*. *Appl Environ Microbiol* **77**, 6687-6690.

Pool, W. (2008). Engineering of sugar metabolism in *L. lactis*. Ph.D. thesis, Groningen Biomolecular Sciences and Biotechnology Institute, Faculty of Mathematics and Natural Sciences, University of Groningen, Groningen.

Pool, W.A., Neves, A.R., Kok, J., Santos, H., and Kuipers, O.P. (2006). Natural sweetening of food products by engineering *Lactococcus lactis* for glucose production. *Metab Eng* **8**, 456-464.

Poolman, B., and Konings, W.N. (1993). Secondary solute transport in bacteria. *Biochim Biophys Acta* **1183**, 5-39.

Poolman, B., Smid, E.J., Veldkamp, H., and Konings, W.N. (1987). Bioenergetic consequences of lactose starvation for continuously cultured *Streptococcus cremoris*. *J Bacteriol* **169**, 1460-1468.

Postma, P.W., Lengeler, J.W., and Jacobson, G.R. (1993). Phospho*enol*pyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* **57**, 543-594.

Ramos, A., Neves, A.R., Ventura, R., Maycock, C., Lopez, P., and Santos, H. (2004). Effect of pyruvate kinase overproduction on glucose metabolism of *Lactococcus lactis*. *Microbiology* **150**, 1103-1111.

Rees, D.C., Johnson, E., and Lewinson, O. (2009). ABC transporters: the power to change. *Nat Rev Mol Cell Biol* **10**, 218-227.

Saier, M.H., Jr. (2000). Families of transmembrane sugar transport proteins. *Mol Microbiol* **35**, 699-710.

Santos, H., and Turner, D.L. (1986). Characterization of the improved sensitivity obtained using a flow method for oxygenating and mixing cell suspensions in NMR. *J Magnet Reson* **68**, 345-349.

Schleifer, K.H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W., and Amann, R. (1995). Application of molecular methods for the classification and identification of lactic acid bacteria. *International Dairy Journal* **5**, 1081-1094.

Schleifer, K.H., Kraus,J., Dvorak,C., Kilpper-Bälz,R., Collins,M.D., and Fischer,W. (1985). Transfer of *Streptococcus lactis* and related species to the genus *Lactococcus* gen. nov. *System Appl Microbiol* **6**, 183-195.

Siezen, R.J., Bayjanov, J., Renckens, B., Wels, M., van Hijum, S.A., Molenaar, D., and van Hylckama Vlieg, J.E. (2010). Complete genome sequence of *Lactococcus lactis* subsp. *lactis* KF147, a plant-associated lactic acid bacterium. *J Bacteriol* **192**, 2649-2650.

Solem, C., Defoor, E., Jensen, P.R., and Martinussen, J. (2008). Plasmid pCS1966, a new selection/counterselection tool for lactic acid bacterium strain construction based on the *oroP* gene, encoding an orotate transporter from *Lactococcus lactis*. *Appl Environ Microbiol* **74**, 4772-4775.

Solem, C., and Jensen, P.R. (2002). Modulation of gene expression made easy. *Appl Environ Microbiol* **68**, 2397-2403.

Sybesma, W., Starrenburg, M., Kleerebezem, M., Mierau, I., de Vos, W.M., and Hugenholtz, J. (2003). Increased production of folate by metabolic engineering of *Lactococcus lactis*. *Appl Environ Microbiol* **69**, 3069-3076.

Teraoka, H., Izui, K., and Katsuki, H. (1974). Phospho*enol*pyruvate carboxylase of *Escherichia coli*. Multiple conformational states elicited by allosteric effectors. *Biochemistry* **13**, 5121-5128.

Termont, S., Vandenbroucke, K., Iserentant, D., Neirynck, S., Steidler, L., Remaut, E., and Rottiers, P. (2006). Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance. *Appl Environ Microbiol* **72**, 7694-7700.

Thompson, J. (1979). Lactose metabolism in *Streptococcus lactis*: phosphorylation of galactose and glucose moieties in vivo. *J Bacteriol* **140**, 774-785.

Thompson, J. (1980). Galactose transport systems in *Streptococcus lactis*. *J Bacteriol* **144**, 683-691.

Thompson, J. (1987). Sugar transport in the lactic acid bacteria. In Sugar transport and metabolism in Gram-positive bacteria (pp 13-38) Ellis Horwood Limited, Chichester, J. Reizer, Peterkofsky, A. (Eds.), ed.

Thompson, J., and Chassy, B.M. (1981). Uptake and metabolism of sucrose by *Streptococcus lactis*. *J Bacteriol* **147**, 543-551.

Thompson, J., and Chassy, B.M. (1985). Intracellular phosphorylation of glucose analogs via the phospho*enol*pyruvate: mannose-phosphotransferase system in *Streptococcus lactis*. *J Bacteriol* **162**, 224-234.

Thompson, J., Chassy, B.M., and Egan, W. (1985). Lactose metabolism in *Streptococcus lactis*: studies with a mutant lacking glucokinase and mannose-phosphotransferase activities. *J Bacteriol* **162**, 217-223.

Vadeboncoeur, C., St Martin, S., Brochu, D., and Hamilton, I.R. (1991). Effect of growth rate and pH on intracellular levels and activities of the components of the phospho*enol*pyruvate: sugar phosphotransferase system in *Streptococcus mutans* Ingbritt. *Infect Immun* **59**, 900-906.

Wang, J., Zhu, J., Bennett, G.N., and San, K.Y. (2011). Succinate production from different carbon sources under anaerobic conditions by metabolic engineered *Escherichia coli* strains. *Metab Eng* **13**, 328-335.

Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M.J., Kuipers, O.P., *et al.* (2007). Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. *J Bacteriol* **189**, 3256-3270.

Williams, A.M., Fryer, J.L., and Collins, M.D. (1990). *Lactococcus piscium* sp. nov. a new *Lactococcus* species from salmonid fish. *FEMS Microbiol Lett* **56**, 109-113.

Wolken, W.A., Lucas, P.M., Lonvaud-Funel, A., and Lolkema, J.S. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis*. *J Bacteriol* **188**, 2198-2206.

Zeikus, J.G., Jain, M.K., and Elankovan, P. (1999). Biotechnology of succinic acid production and markets for derived industrial products. *Appl Microbiol and Biotech* **51**, 545-552.

Zeng, L., and Burne, R.A. (2010). Seryl-phosphorylated HPr regulates CcpA-independent carbon catabolite repression in conjunction with PTS permeases in *Streptococcus mutans*. *Mol Microbiol* **75**, 1145-1158.

Zurbriggen, A., Schneider, P., Bahler, P., Baumann, U., and Erni, B. (2010). Expression, purification, crystallization and preliminary X-ray analysis of the EIICGlc domain of the *Escherichia coli* glucose transporter. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **66**, 684-688.