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Lactose and β -Glucosides Metabolism and Its Regulation in *Lactococcus lactis*: A Review

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1. Introduction

1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, low-GC-content bacteria that comprise 11 bacterial genera, such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and others (Stiles & Holzapfel, 1997). LAB have a generally regarded as safe (GRAS) Food and Drug Administration (FDA) status, and some strains of different LAB species exhibit also probiotic properties (Gilliland, 1989). They are ubiquitous in many nutrient rich environments, such as milk, meat and plant material, and some of them are permanent residents of mainly mammalian intestinal tracts, while others are able to colonize them temporarily. Due to their ability to produce lactic acid as an end product of sugar fermentation, they are industrially important and are used as starter cultures in various food-fermentation processes. The importance of LAB for humans can be appreciated from the estimated 8.5 billion kg of fermented milk produced annually in Europe, leading to human consumption of 8.5×10^{20} LAB (Franz et al., 2010).

Understanding the mechanisms involved in carbohydrate metabolism and its regulation in LAB is essential for improving the industrial properties of these microorganisms. There are several ways to improve the metabolic potential of LAB cells, of which metabolic engineering offers a very efficient and effective tool.

1.2. Genus *Lactococcus*

Lactococci are homofermentative, mesophilic LAB that basically inhabit two natural environments, milk and plants, of which plants seem to constitute the primary niche. Occasionally, there have been reports that *L. lactis* was also isolated from soil, effluent water, the skin of cattle (Klijn et al., 1995), insects (leafhoppers, termites) (Bauer et al., 2000;

Latorre-Guzman et al., 1977; Schultz & Breznak, 1978) and fish (Itoi et al., 2008, 2009; Pérez et al., 2011). Adaptation of lactococcal strains from plants to the dairy environment has caused the loss of some functions, resulting in smaller chromosomes and acquisition of genes (often plasmidic) important for growth in milk (Kelly et al., 2010).

Since *Lactococcus lactis* was first described in 1919 (Orla-Jensen, 1919), its taxonomy has changed repeatedly and still is confusing in some aspects. This group of bacteria, previously designated lactic streptococci, was placed in the new *Lactococcus* taxon in 1985 (Schleifer et al., 1985). The current taxonomy of *L. lactis* is based on phenotype and includes four subspecies (*lactis*, *cremoris*, *hordniae*, and the newly identified subsp. *tractae*) and one biovar (subsp. *lactis* biovar *diacetylactis*) (Schleifer et al., 1985; van Hylckama Vlieg et al., 2006; Pérez et al., 2011; Rademaker et al., 2007). Among them, only *L. lactis* subsp. *hordniae* and subsp. *tractae* have never been isolated from dairy products. The *lactis* and *cremoris* phenotypes are distinguished on the basis of several basic criteria, such as: arginine and maltose utilization, decarboxylation of glutamate to γ -aminobutyric acid (GABA), and 40°C, 4% NaCl and pH 9.2 tolerance. *L. lactis* subsp. *cremoris* strains are reported to be negative for all of these features (Nomura et al., 1999; Schleifer et al., 1985). Moreover, the biovar *diacetylactis* strains are able to metabolize citrate, which is converted to diacetyl, an important aroma compound. Additionally, numerous genetic studies (DNA–DNA hybridization, 16S rRNA and gene sequence analysis) of *L. lactis* isolates of dairy and plant origin have revealed the existence among them of two main genotypes that have also been called *L. lactis* subsp. *lactis* (*lactis* genotype) and *L. lactis* subsp. *cremoris* (*cremoris* genotype). Furthermore, it has been demonstrated that the genotype and phenotype do not always correspond within one isolate, thus introducing a degree of disorder into the taxonomy of this species (Tailliez et al., 1998). It has been observed that within the group of *cremoris* genotype, strains with both *lactis* (MG1363) and *cremoris* (SK11) phenotypes may occur, and, likewise, within the group of *lactis* genotype there are ones with *lactis* (KF147) as well as biovar *diacetylactis* (IL594) phenotypes (Bayjanov et al., 2009; Kelly et al., 2010; Nomura et al., 2002; Rademaker et al., 2007; Tanigawa et al., 2010). Hence, the *L. lactis* has an atypical taxonomic structure with two phenotypically distinct groups, such as *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, which may belong to two distinct genotype groups. As a result, in order to sufficiently describe the individual strains, it is necessary to specify both the genotype (*cremoris* or *lactis*) and the phenotype (*cremoris*, *diacetylactis*, or *lactis*).

Strains belonging to *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* together with a diverse assortment of other LAB are widely used as dairy starters for the production of a vast range of fermented dairy products, including various types of cheeses, sour cream, buttermilk and butter (Daly, 1983; Davidson et al., 1996). In the dairy industry, the *lactis* subspecies are better for making soft cheeses and the *cremoris* subspecies for the hard ones. Overall, it is generally accepted that the *L. lactis* subsp. *cremoris* strains make better quality products than *L. lactis* subsp. *lactis* because of their important contribution to flavour development via their unique metabolic mechanisms (Salama et al., 1991; Sandine, 1988).

During growth in milk, the primary function of *L. lactis* is rapid conversion of lactose to lactic acid, which provides preservation of the fermented product by preventing growth of

pathogenic and spoilage bacteria, it supports curd formation, and creates optimal conditions for ripening. Further, due to their proteolytic activity and amino acid conversion, lactococci contribute to the final texture (moisture, softness) and flavour of dairy products (Smit et al., 2005). Many of lactococcal functions vital for successful fermentations are borne on plasmids, which are a common feature in lactococci, even in strains isolated from non-dairy sources (Davidson et al., 1996). For example, specific plasmid-borne genes encode proteins involved in lactose transport and metabolism and in hydrolysis and utilization of casein (Davidson, et al., 1996; McKay, 1983). Hence, there is considerable selective pressure on dairy strains to retain these plasmids, since plasmid-cured derivatives grow poorly in milk. Since plasmids are mobile elements, they can be readily exchanged among different strains (via conjugal transfer) (Gasson, 1990).

Due to its industrial importance *L. lactis* has become the best studied LAB, and although most studies have been performed on a small number of laboratory strains of dairy origin, it is regarded as a model organism for this bacterial group. A number of genome sequences of *L. lactis* strains are available, including strains from *L. lactis* subsp. *lactis*, such as IL1403, KF147 and CV56, as well as strains from *L. lactis* subsp. *cremoris*, such as MG1363, A76, NZ9000 and SK11 (according to <http://www.ncbi.nlm.nih.gov/genome/>). Among them, *L. lactis* subsp. *lactis* IL1403 (Chopin et al., 1984) and *L. lactis* subsp. *cremoris* MG1363 (Gasson, 1983) are the most important laboratory strains, and they can be distinguished by differences in specific DNA sequences, including those encoding 16S rRNA (Godon et al., 1992), and by their genome organization (Le Bourgeois et al., 1995). These two strains are plasmid-cured derivatives of the dairy starter strains IL594 (IL1403) and NCDO 712 (MG1363) respectively, and due to their industrial importance, their metabolism, physiology and genetics have been extensively studied over the past years. Both belong to *L. lactis* subsp. *lactis* phenotypically, but the parent strain of IL1403 has a citrate permease plasmid (Górecki et al., 2011) and is able to metabolize citrate, placing it with *L. lactis* subsp. *lactis* biovar diacetylactis, whereas MG1363 has a *lactis* phenotype and a *cremoris* genotype (Kelly et al., 2010). Despite their physiological and 16S rRNA gene sequence similarities, they share only about 85% chromosomal sequence identity, which is comparable to the genetic distance between *Escherichia coli* and *Salmonella typhimurium* (McClelland et al., 2001; Salama et al., 1991; Wegmann et al., 2007). A derivative of MG1363 was created by the integration of the *nisRK* genes (involving the “NICE” system for nisin-controlled protein overexpression) into the *pepN* gene, yielding *L. lactis* NZ9000 (Kuipers et al., 1998).

2. Lactose metabolism

Most microorganisms have adapted to growth in milk habitat due to acquisition of the ability to use its most abundant sugar, lactose, as a carbon source. This disaccharide consists of a galactose moiety linked at its C₁ via a β -galactosidic bond to the C₄ of glucose. Because of the efficiency and economic importance of its fermentation, a large number of studies have focused on the utilization of lactose by LAB.

Uptake of lactose into a bacterial cell can be mediated by several pathways, such as the lactose-specific phosphotransferase system (*lac*-PTS), ABC protein-dependent systems and

secondary system transporters like lactose-galactose antiporters and lactose-H⁺ symport systems (de Vos & Vaughan, 1994). While ABC protein-dependent lactose transport has been demonstrated only in non-LAB, Gram-negative *Agrobacterium radiobacter* (Williams et al., 1992), the *lac*-PTS as well as secondary lactose transport systems have been described for many LAB species.

2.1. Lactose-specific phosphotransferase systems (*lac*-PTS)

Although LAB used as starter cultures may also convert pyruvate to a variety of end products, these pathways are not expressed during lactose fermentation, which is homolactic in most strains (Cocaign-Bousquet et al., 2002; Neves et al., 2005). Since the primary function of LAB in dairy fermentations is the conversion of lactose to lactic acid, the industrial strains are primarily selected on the basis of their ability for its rapid, homolactic fermentation (de Vos & Simons, 1988).

Starter lactococcal strains transport lactose exclusively by the most abundant in LAB uptake system for various sugars - the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). The *lac*-PTS has a very high affinity for this sugar and is bioenergetically the most efficient system since one lactose molecule is translocated and phosphorylated in a single step, at the expense of a single ATP equivalent. Concomitantly with transport, PTS catalyzes the phosphorylation of the incoming sugar. Phosphoenolpyruvate is the first phosphoryl donor, which phosphorylates Enzyme I (EI), and then the phosphoryl group is transferred in sequence to HPr, EIIA, EIIB, and finally, via transmembrane porter (EIIC), to the transported sugar (Lorca et al., 2010). After translocation via *lac*-PTS, lactose is hydrolyzed by P- β -galactosidase to glucose and galactose-6-P. While glucose enters the Embden-Meyerhof-Parnas glycolytic pathway through phosphorylation by glucokinase, galactose-6-P, before it also enters the glycolytic pathway, is further metabolized via the D-tagatose-6-P (Tag-6P) pathway. This involves three enzymes: (i) galactose-6-P isomerase (LacAB); (ii) tagatose-6-P kinase (LacC); and (iii) tagatose-1,6-diphosphate aldolase (LacD). The resulting triosephosphates (glyceraldehydes-3-P and dihydroxyacetone-P) are further metabolized via glycolysis. The operons engaged in this rapid, homolactic lactose fermentation are usually plasmid-located (*lac*-plasmids) and, in addition to the genes for the *lac*-PTS proteins and P- β -galactosidase, contain genes coding for the enzymes of the Tag-6P pathway. Their transcription is regulated by various repressors, with tagatose-6-P being the molecular inducer in *L. lactis* (van Rooijen et al., 1991).

It is believed that plasmid-encoded ability for rapid lactose fermentation characteristic for dairy strains was recently acquired by wild-type plant strains, as a result of their adaptation to milk-environment (Kelly et al., 2010).

2.2. Lactose permease- β -galactosidase systems

Another strategy developed by LAB for lactose metabolism depends on its uptake via secondary transport systems. These systems transport lactose in an unphosphorylated form via specific permeases belonging to the LacS subfamily (TC No. 2.A.2.2.3) of the 2.A.2 glycoside-pentoside-hexuronide (GPH) family (Saier, 2000). Carriers of the LacS subgroup

are chimeric in nature: at their carboxy terminal end they contain an approximately 160 amino acid hydrophilic extension homologous to the EIIA domains of PTS. Thus, lactose transport is controlled by HPr-dependent phosphorylation (Gunnewijk et al., 1999; Gunnewijk & Poolman, 2000a; Gunnewijk & Poolman, 2000b). Due to this additional domain these lactose permeases are larger than the other carriers from the GPH family, which are generally about 500 amino acids in length. Depending on the organism, LacS can mediate lactose transport coupled to proton symport or by antiport with galactose. Following its import, lactose is hydrolyzed by β -galactosidase (David et al., 1992; Vaughan et al., 1996) yielding glucose and galactose. The glucose moiety is further metabolized via glycolysis, whereas the galactose moiety follows different pathways depending on the particular LAB. While some thermophilic strains of LAB (e.g., *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) are known to release the galactose moiety of lactose into the medium, other LAB (e.g., *Lactobacillus helveticus*, *Leuconostoc lactis* and *Streptococcus salivarius*) metabolize this saccharide via the Leloir pathway (de Vos, 1996; Poolman, 1993; Vaughan et al., 2001). This pathway was one of the first central metabolic pathways to be discovered, by L. F. Leloir and coworkers in the early 1950s. It includes the key enzyme galactokinase (GalK), and hexose-1-P uridylyltransferase (GalT) plus UDP-glucose 4-epimerase (GalE), all of which are involved in the conversion of galactose to glucose-1P. The generated glucose-1P, after conversion to glucose-6P by phosphoglucumutase, enters the glycolytic pathway. Aldose-1-epimerase, a mutarotase (GalM), is an additional, more recently characterized enzyme required for rapid galactose metabolism (Bouffard et al., 1994; Mollet & Pilloud, 1991; Poolman et al., 1990). GalM catalyses the interconversion of the α - and β -anomers of galactose. This enzyme was found to be essential for efficient lactose utilization in *E. coli* since cleavage of this β -galactoside by β -galactosidase yields glucose and β -D-galactose, the latter being the sole substrate for GalK (Bouffard et al., 1994).

The existence of genes encoding components of the lactose permease- β -galactosidase system seems to be limited among the *L. lactis* strains as they have been identified only in the genomes of the dairy-derived strain IL1403 (Bolotin et al., 2001), non-dairy NCDO2054 (Vaughan et al., 1998) and KF147 isolated from mung bean sprouts (Siezen et al., 2010). Remarkably, in addition to galactose genes of the Leloir pathway cluster, these strains contain genes needed for lactose assimilation, such as *lacZ* (β -galactosidase) and *lacA* (thiogalactoside acetyltransferase), arranged in an identical layout. Directly upstream of the aforementioned genes required for lactose hydrolysis and subsequent galactose conversion, there is the gene encoding the LacS permease for sugar uptake.

Some details concerning the role of the lactose permease- β -galactosidase system in lactose utilization have been reported for the slow lactose fermenter - *L. lactis* NCDO2054 (Vaughan et al., 1998), and for the devoid of the *lac*-plasmid, essentially lactose-negative *L. lactis* IL1403 strain (starts to utilize lactose slowly after approximately 40 h of incubation) (Aleksandrzyk-Piekarczyk et al., 2005). Since these strains possess the complete lactose permease- β -galactosidase system and an active Leloir pathway, it seems odd that they are barely capable of lactose metabolism. In the case of *L. lactis* NCDO2054, which can accumulate a high intracellular concentration of lactose-6-phosphate by using an efficient *lac*-PTS and

possesses low-level P- β -galactosidase activity, it has been suggested that the slow fermentation of lactose may be due to this rate-limiting P- β -galactosidase activity and the inhibitory effect of the accumulated lactose-6-phosphate (Bissette & Anderson 1974; Crow & Thomas, 1984). However, other explanations of lactose fermentation problem can be envisaged: (i) lactose transport is inefficient due to low affinity of LacS for lactose or (ii) the strains lack a functional β -galactosidase. Indeed, the *lacS* gene of *L. lactis* IL1403 is almost identical to that of *L. lactis* NCDO2054, but also to *galP* of the lactose-negative *L. lactis* MG1363 strain (Grossiord et al., 2003). These permeases belong to the same subfamily (TC No. 2.A.2.2.3 according to the Transporter Classification Database: <http://www.tcdb.org/>; Saier, 2000), which includes transporters specific for galactose uptake, in contrast to LacS permeases of another subfamily (TC No. 2.A.2.2.1) with a proven high lactose-transport rate. The lack of LacS involvement in lactose transport is confirmed by the fact that disruption of *lacS* in *L. lactis* IL1403 had a minor effect on lactose assimilation (Aleksandrak-Piekarczyk et al., 2005). Another indispensable factor in lactose assimilation, the β -galactosidase enzyme, is also encoded by the genomes of *L. lactis* IL1403 and NCDO2054 strains. In spite of the high similarity in the protein level of both enzymes, β -galactosidase of *L. lactis* NCDO2054, in contrast to the one of *L. lactis* IL1403 (Aleksandrak-Piekarczyk et al., 2005), seems to be highly active and strongly regulated (Griffin et al., 1996). It has been suggested that the *lacZ* gene of *L. lactis* IL1403 may not be expressed or the encoded enzyme may be inactive since this strain does not exhibit β -galactosidase activity (Aleksandrak-Piekarczyk et al., 2005). Furthermore, the *in trans* complementation of chromosomal *lacZ* by an active β -galactosidase in *L. lactis* IL1403 did not improve its ability for lactose assimilation, indicating that the lack of β -galactosidase activity is not the only obstacle in its ability to efficiently ferment lactose (unpublished personal observations).

Taken together, it seems that in *L. lactis* strains lactose permease- β -galactosidase systems play a minor role in lactose assimilation or function under certain environmental conditions. It appears that the major obstacle is the galactose-specific LacS permease, which shows only weak affinity for lactose and functions almost only in transport of galactose (Fig. 1). This thesis is confirmed by the study of Solem et al. (2008), in which an efficient lactose transporter (LacS; TC No. 2.A.2.2.1) and β -galactosidase (LacZ), encoded by the *lacSZ* operon, were introduced from lactose-positive *S. thermophilus* into the lactose-negative strain *L. lactis* MG1363, devoid of lactose permease- β -galactosidase system. As a result, fast-growing lactose-positive mutant strains were obtained. This shows that addition of the LacSZ system containing LacS with a proven high lactose-transport rate can strongly increase the lactose-transport capacity in *L. lactis*.

3. Metabolism of β -glucosides

In addition to dairy environment, plant surfaces and fermenting plant material are also important ecosystems occupied by *L. lactis*. With regard to fermentation, lactococcal strains usually occur there only at the beginning of this process, to be later replaced by microorganisms more resistant to low pH values (Kelly & Ward, 2002; Kelly et al., 1998). The majority of plant-associated strains belong to *L. lactis* subsp. *lactis*, whereas *L. lactis*

subsp. *cremoris* is typical for dairy fermentations (Kelly & Ward, 2002; Kelly et al., 1998). In comparison to the dairy environment, fermenting plant material differs highly with respect to chemical composition, exhibiting, for instance, much lower protein concentration and wider availability of carbohydrates other than lactose. The ability of plant-associated *L. lactis* subsp. *lactis* strains to utilize such a large variety of plant carbohydrates is reflected in their genomes and sugar fermentation capabilities. Comparison between milk- and plant-associated lactococcal strains clearly shows that the latter possess a larger number of genes involved in transport and metabolism of carbohydrates, resulting in their increased sugar fermentation capabilities (Siezen et al., 2008).

Besides lactose, the PTS systems can also transport various other carbohydrates, including sugars widely distributed in plants, namely β -glucosides, like e.g. amygdalin, arbutin, cellobiose, esculin, gentobiose and salicin (Tobisch et al., 1997). Except for amygdalin, these sugars are composed of two molecules joined by the β -glucosidic bond, of which at least one is glucose. The best known example of this group is cellobiose, the structural unit of one of the most abundant renewable polymers on earth – cellulose, and also the main product in its enzymatic hydrolysis (Teeri, 1997). Unlike most of other β -glucosides (aryl- β -glucosides e.g., arbutin, amygdalin, esculin, and salicin), which are composed of a single glucose molecule and respective aglycone, cellobiose consists of two glucose molecules linked via a $\beta(1-4)$ bond.

It is well known from sugar fermentation characteristics that *L. lactis* strains of different origin can utilize a variety of β -glucosides (e.g., Aleksandrzyk-Piekarczyk et al., 2011; Bardowski et al., 1995; Fernández et al., 2011; Siezen et al., 2008). The metabolic potential for catabolism of these sugars can be chromosomally encoded by more than one genetic system, as was shown for *L. lactis* IL1403. Eight genes, which encode proteins homologous to EII proteins of β -glucoside-dependent PTS, involved in the uptake and phosphorylation of β -glucosides have been found throughout the *L. lactis* IL1403 chromosome (Bolotin et al., 2001). Three of them encode the three-domain EIIABC PTS components (PtbA, YedF and YleE), another three, EIIC permeases (CelB, PtcC and YidB), one an EIIA component (PtcA) and one an EIIB component (PtcB). CelB, PtcA, PtcB, PtcC and YidB are members of the Lac family (TC No. 4.A.3), which includes several lactose porters of Gram-positive bacteria as well as the *E. coli* and *Borrelia burgdorferi* N,N'-diacetylchitobiose (Chb) porters (according to <http://www.tcdb.org/>). The involvement of CelB and CelB/PtcC permeases in cellobiose transport has been experimentally confirmed in *L. lactis* IL1403 and MG1363, respectively (Aleksandrzyk-Piekarczyk et al., 2011; Campelo et al., 2011). Although *L. lactis* IL1403 has such a large number of β -glucosides-specific PTS systems, CelB is the only permease operative in cellobiose uptake in this strain (Aleksandrzyk-Piekarczyk et al., 2011) (Fig. 1), whereas in *L. lactis* MG1363 also another PTS permease, namely PtcC, seems to participate in the transport of this sugar, albeit to a much lesser extent than CelB (Campelo et al., 2011). It has been proposed that the observed low expression of the *ptcC* gene may be the result of repression by carbon catabolite control protein A (CcpA) as mutations in its binding site (catabolite responsive element - *cre*) in the *ptcC* promoter region led to high upregulation of this gene in strain NZ9000 compared to strain MG1363, even under repressive conditions (Linares et al., 2010).

On the other hand, the EIIAB components, namely PtcA and PtcB, seem to be more versatile, being involved in the metabolism of numerous sugars (arbutin, cellobiose, glucose, lactose, salicin) in *L. lactis* (Aleksandrak-Piekarczyk et al., 2011; Castro et al., 2009; Pool et al., 2006). No other PTS systems dedicated to transport of other β -glucosides have yet been described in detail in any *L. lactis* strain. However, according to unpublished preliminary data, the PtbA protein appears to be involved in the transport of arbutin, esculin and salicin, but not cellobiose, in *L. lactis* IL1403 (unpublished personal observation) (Fig. 1). In this strain, inactivation of the *ptbA* gene led to serious defects in growth in medium supplemented with each of these sugars (unpublished).

After translocation by PTS through the bacterial membrane, the P- β -glucoside sugar is cleaved by P- β -glucosidase into glucose and glucose-6P or the respective aglycon (Tobisch et al., 1997). There are plenty of genes encoding P- β -glucosidases present in *L. lactis* chromosomes sequenced so far. Their large number is probably the result of adaptation of these bacteria to life on plants with abundant where β -glucosides. However, the data concerning their involvement in β -glucosides assimilation are rather scarce in scientific literature. It has only been demonstrated that a P- β -glucosidase, BglS, is responsible for hydrolysis of cellobiose, but not of salicin in *L. lactis* IL1403 (Aleksandrak-Piekarczyk et al., 2005) (Fig. 1). On the other hand, no function has been attributed to another P- β -glucosidase encoded by the *bglA* gene, and forming one operon with *ptcC*. According to unpublished results, the disruption of *bglA* did not alter growth of the IL1403 mutant strain in medium supplemented with a wide array of sugars (unpublished personal analysis).

Expression of β -glucosides' catabolic genes can be controlled by various regulatory mechanisms. Among them, catabolite repression (Aleksandrak-Piekarczyk et al., 2005, 2011; Zomer et al., 2007) and transcriptional antitermination through the BglR protein (Bardowski et al., 1994) were shown to be operational in *L. lactis*. The antitermination mechanism allows for expression of β -glucoside-specific genes in the absence of a metabolically preferred carbon source, such as glucose (Rutberg, 1997). It is believed that antiterminator proteins act by binding to a ribonucleic antiterminator (RAT) site at a specific mRNA secondary structure to prevent the formation of a hairpin terminator structure that would otherwise terminate transcription (Aymerich & Steinmetz, 1992; Rutberg, 1997). The binding of the antiterminator protein to the mRNA permits transcription through the sequestered terminator sequence into a β -glucoside-specific operon that is not normally transcribed. The function of BglR has been studied earlier in *L. lactis* IL1403, and it was shown to be involved in the activation of assimilation of β -glucosides such as arbutin, esculin and salicin, except for cellobiose (Bardowski et al., 1994; 1995) (Fig. 1). Inspection of the *L. lactis* IL1403 genome sequence downstream of *bglR* revealed the presence of two genes, *ptbA* and *bglH*, encoding proteins homologous to a putative three-domain EIIABC PTS component specific for the assimilation of β -glucosides, and P- β -glucosidase, respectively. Upstream of *bglR*, a putative *cre*-box (differing from the *cre* consensus by one nucleotide), a putative promoter sequence and a RAT sequence were identified. This RAT sequence has been reported previously (Bardowski et al., 1994, 1995) to be involved in the autoregulation of BglR. This sequence partially overlapped a putative *rho*-independent

terminator, which comprised six nucleotides at the 3' end of the RAT. The *ptbA* gene is located 141 nt downstream of *bglR*. *In silico* sequence analysis revealed that the *ptbA* gene is also preceded by a DNA sequence highly similar to the RAT consensus sequence, suggesting that the regulation of *ptbA* expression may involve the BglR-mediated antitermination mechanism (unpublished personal analysis). Moreover, the short intergenic DNA region (47 nt) between *ptbA* and the next gene (*bglH*), plus the lack of an obvious hairpin structure or a promoter sequence strongly suggest that these two genes might be cotranscribed, and thus undergo common BglR-mediated regulation (unpublished) (Fig. 1).

4. Alternative lactose utilization system and its interconnection with cellobiose assimilation

The existence in several lactococcal strains devoid of *lac*-plasmids of cryptic lactose transport and catabolism systems has already been suggested in earlier studies (Anderson & McKay, 1977; Cords & McKay, 1974; de Vos & Simons, 1988; Simons et al., 1993). The presence in *L. lactis* of chromosomally-encoded lactose permease has been proposed since introduction of the *E. coli lacZ* gene into a lactose-deficient *L. lactis* strain restored its ability to utilize lactose (de Vos & Simons, 1988). Moreover, P- β -galactosidase activities have also been detected in strains cured of their lactose plasmids, suggesting the presence of chromosomally-encoded cryptic *lac*-PTS(s) (Anderson & McKay, 1977; Cords & McKay, 1974). However, it was suggested that these PTSs are not specific for lactose, but rather for the translocation of other sugars (e.g., β -glucosides), and lactose could be transported alternatively. This hypothesis was supported by observations suggesting that a putative P- β -glucosidase, involved in cellobiose hydrolysis, is probably also involved in lactose-6-P cleavage in *L. lactis* strain ATCC7962 (Simons et al., 1993). This seems reasonable, as according to <http://www.tcdb.org/>, PTS lactose transporters belong to the Lac family (TC No. 4.A.3) and porters of this family have broad substrate specificity. Besides lactose, they can also transport aromatic β -glucosides and cellobiose.

Until recently (Aleksandrak et al., 2000; Aleksandrak-Piekarczyk et al., 2005, 2011; Kowalczyk et al., 2008), little information on the organization in *L. lactis* strains of chromosomal alternative lactose utilization genes has been available. It was shown that in *lac*-plasmid-free, and thus lactose-negative *L. lactis* IL1403, the ability to assimilate lactose can be induced in two ways: (i) by the presence of cellobiose or (ii) by inactivation of CcpA (Aleksandrak et al., 2000; Aleksandrak-Piekarczyk et al., 2005). The CcpA protein is a member of the LacI-GalR family of bacterial repressors and exists only in Gram-positive bacteria (Weickert & Adhya, 1992). It exerts its regulatory role in carbon catabolite repression (CCR) by binding to DNA sites called *cres*, which occur in the vicinity of CcpA-regulated genes (Weickert & Chambliss, 1990). In *L. lactis* the known targets of CcpA are the *gal* operon for galactose utilization (Luesink et al., 1998), the *fru* operon for fructose utilization (Barrière et al., 2005), the *ptcABC* operon for cellobiose utilization (Zomer et al., 2007), and *cel-lac* genes for cellobiose and lactose utilization (Aleksandrak-Piekarczyk et al., 2011). Thus, one could speculate that in *L. lactis* IL1403 cellobiose-inducible chromosomal

alternative lactose utilization genes are under the negative control of CcpA, and, therefore, inactivation of the *ccpA* gene could result in their derepression and ability to assimilate lactose by the IL1403 *ccpA* mutant.

Further studies of Aleksandrak-Piekarczyk et al. (2005, 2011) and Kowalczyk et al. (2008) provided details on interconnected metabolism of β -glucosides (cellobiose) and β -galactosides (lactose) and its variable regulation in *L. lactis* IL1403. Several genes have been implicated in coupled cellobiose and lactose assimilation in *L. lactis* IL1403, such as *bglS* and *celB*, *ptcA* and *ptcB*, encoding proteins homologous to P- β -glucosidase and EII components of cellobiose-specific PTS, respectively (Fig. 1). It has been shown that in *L. lactis* IL1403 the cellobiose-specific PTS system, comprising of *celB*, *ptcB* and *ptcA*, is also able to transport lactose because cellobiose-specific permease CelB has also an affinity for lactose, and, moreover, is the only permease involved in lactose uptake (Aleksandrak-Piekarczyk et al., 2011). Furthermore, internalized lactose-P is hydrolyzed exclusively by BglS – an enzyme with dual P- β -glucosidase and P- β -galactosidase activity, and high affinity for cellobiose (Aleksandrak-Piekarczyk et al., 2005) (Fig. 1). Thus, BglS activity generates glucose and galactose-P molecules. Glucose enters the Embden-Meyerhof-Parnas glycolytic pathway through phosphorylation by glucokinase, whereas galactose-P requires dephosphorylation performed by an unidentified phosphatase or phosphohexomutase, before entering the Leloir pathway (Neves et al., 2010) (Fig. 1). Moreover, this alternative lactose utilization system has been shown to be tightly controlled by CcpA-directed negative regulation (Fig. 1), since inactivation of the *ccpA* gene led to derepression of *bglS*, *celB*, *ptcA* and *ptcB* and *L. lactis* IL1403 *ccpA* mutant ability to assimilate lactose (Aleksandrak-Piekarczyk et al., 2011). In addition to CcpA-mediated repression, the *celB* and *bglS* genes are specifically activated by cellobiose, as its presence leads to an increase in their transcription. This phenomenon has not been observed when other sugars, such as glucose, galactose or salicin, were used as carbon sources (Aleksandrak-Piekarczyk et al., 2011). Preliminary results suggest that a hypothetical transcriptional regulator, namely YebF, could be engaged in this cellobiose-dependent activation of *celB* and *bglS* (Aleksandrak-Piekarczyk et al., 2011; unpublished personal analysis) (Fig. 1). The YebF protein belongs to the RpiR family of phosphosugar binding proteins (Sorensen & Hove-Jensen, 1996), and, in addition to its sugar binding domain (SIS), it has a putative helix-turn-helix (HTH) DNA-binding domain. In addition to *yebF* mutant ferment lactose inability (Aleksandrak-Piekarczyk et al., 2005), inactivation of the *yebF* gene in IL1403 resulted in inability to grow on cellobiose (unpublished personal analysis), suggesting the gene's requirement in both cellobiose and lactose assimilation. Further studies on this phenomenon in *L. lactis* are needed to address it in greater detail.

When cellobiose is available, it activates the cellobiose-specific PTS transport system, comprising CelB, PtcB and PtcA proteins, and *L. lactis* IL1403 is able to grow on cellobiose and lactose. This growth is supported by the activity of cellobiose-inducible BglS protein, which splits lactose-P into galactose-P and glucose. Then, after the dephosphorylation step, galactose is further metabolized through the Leloir pathway, while glucose enters glycolysis. Therefore, inactivation of the *ccpA* gene results in derepression of the cellobiose-specific PTS transport system and also of the *bglS* gene, which in turn enable the IL1403 strain to grow on lactose.

Figure 1. Schematic representation of the proposed mechanism of chromosomally-encoded lactose, cellobiose-inducible lactose and β -glucosides metabolism and of its regulation in *L. lactis* IL1403. In this model the key elements are the CelB, PtcB, PtcA, BglS and PtbA proteins. In the presence of glucose, IL1403 is unable to assimilate either lactose or β -glucosides. Under these conditions, these catabolic systems are either repressed by the CcpA protein and/or are not induced by the BglR activator.

Besides cellobiose, other β -glucosides like arbutin, esculin and salicin are transported by the PtbA-mediated PTS system. In the absence of any of these three sugars, *ptbA* expression is not induced by the inactive the phosphorylated BglR antiterminator protein. Once a β -glucoside is available, BglR becomes dephosphorylated and active, inducing the expression of the *ptbA* gene. The PtbA protein transports, with concomitant phosphorylation, arbutin, esculin and salicin, which are then probably hydrolyzed by BglH, a P- β -glucosidase, encoded by a gene located downstream of and in the same operon as the *ptbA* gene.

It is also proposed in this model that LacS is not engaged in lactose internalization and its function is limited to galactose transport.

5. Conclusions

Despite the fact that the metabolism of lactose and β -glucosides is very important for the biotechnological processes catalysed by *L. lactis*, thorough studies of the chromosomally encoded features enabling use of these carbon sources were so far rather scarce. The reason for this could be the fact that *L. lactis* demonstrates a very large and complex metabolic capability towards carbohydrates used as carbon and energy sources, and, moreover, that this genetic potential is tightly regulated by various environmental and intracellular factors. It seems that the main obstacle in studies on the complicated

mechanisms involved in assimilation of β -glycoside sugars was the lack of complex data specifying the sequences of genes potentially involved in the metabolism of these sugars and its regulation. Indeed, recent access to the genomic sequences of some these bacteria greatly advanced the research on the metabolism of various β -glycosides. As expected, the results of sequencing of lactococcal genomes and genes annotations confirmed that there are numerous genes encoding potential β -glucosides-specific transport systems and β -glucosidases, sometimes with dual activities. And, to complicate the matter even further, the analysis of the list of genes annotated in *L. lactis* leads to over a hundred transcriptional regulators. A relatively large number of them may be related to carbon metabolism control. These regulators, together with signals modulating their activity, and the controlled genes form a regulatory network that is necessary for sensing the environmental conditions and adjusting the catabolic capacities of the cell.

Detailed knowledge of sugar metabolism and the regulators controlling gene expression in *Lactococcus lactis* may contribute to the improvement of mechanisms controlling significant cellular processes in these bacteria. In the case of industrial microorganisms, acting on the defined regulatory network may drastically affect the properties of the bacteria and have an impact on bioprocesses.

Lastly, is shown as an example that by the use of a simple microbiological screen, it is possible and worthwhile to modify the metabolic potential of lactococcal strains initially unable to assimilate lactose. By inactivation of the *ccpA* gene or induction of particular genes by supplementation of the medium with cellobiose and thus activation of YebF, it is possible to turn on an alternative lactose assimilation pathway in *L. lactis* IL1403. In contrast to plasmid-located *lac*-operons, the *cel-lac* system is within the chromosome, resulting in a stable, highly adapted strain, potentially valuable for the dairy industry.

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