Review of lactose and galactose metabolism in Lactic Acid Bacteria dedicated to expert genomic annotation

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ABSTRACT

Background

The term LAB (Lactic Acid Bacteria) relates to the metabolic capabilities of microorganisms to ferment various carbohydrates predominantly into lactic acid. LAB are involved in a large number of industrial food fermentations. Their primary contribution is rapid acid production and acidification of foods. The ability of starter cultures to metabolize lactose and the resulting galactose is therefore a major aspect of starter selection. Genome sequencing and the use of microbial genomics are powerful tools to rapidly screen for functions of interest in the starter culture industry.

Scope and approach

The annotation of the metabolic pathway encoding genes requires thorough knowledge of the literature and a vast comprehension of the metabolic pathways and of the level of genetic variability that LAB can exhibit. In this review an overview of lactose and galactose metabolism as a basis for expert genomic annotation is presented. It includes a detailed literature overview of the mechanism of internalization, subsequent metabolization of these carbohydrates and the underlying genetics. In addition, a comparative genomic analysis of genes related to the Leloir and Tagatose-6-P pathways is described in 237 LAB and other related Gram-positive genomes with low GC content available in the MicroScope platform.

Key findings and Conclusions

This analysis delineates the extent of genetic variability that can be expected for future genomic investigation of LAB regarding lactose and galatose metabolism.

Keywords: lactose; galactose; genome annotation; Leloir pathway; Tagatose-6Phosphate pathway; Lactic Acid bacteria

1. Introduction

The term LAB (Lactic Acid Bacteria) relates to the metabolic capabilities of microorganisms to ferment various carbohydrates predominantly into lactic acid (Klaenhammer, Barrangou, Buck, Azcarate-Peril, & Altermann, 2005; Liu, 2003). LAB form a heterogeneous group of microorganisms that belong to the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 2004; Pot, 2008; Stackebrandt & Teuber, 1988; Stiles & Holzapel, 1997). LAB are naturally found in a variety of natural habitats, including plants, meat and milk environment. They are involved in a large number of industrial food fermentations where they are used as starter and adjunct cultures. Their primary contribution is rapid acid production and acidification of foods, but metabolic processes accompanying the growth of LAB impact also flavor, nutrition, and texture quality of a variety of fermented foods (Axelsson, 2004; Klaenhammer et al., 2005).

Lactose is a disaccharide consisting of the combination of two simple sugars, α/β -D-glucose and β -D-galactose, linked by a β (1-4) glycosidic bond and is the main carbon source in milk (Kumar, Weatherly, & Beaman, 1991). Utilization of lactose is mainly related to the fermentation processes by LAB and it is well documented (Cocaign-Bousquet, Garrigues, Loubiere, & Lindley, 1996; Kandler, 1983; Leroy & De Vuyst, 2004; Neves, Pool, Kok, Kuipers, & Santos, 2005; Wu, Cheung, & Shah, 2015). Galactose utilization is important at the industrial level where its accumulation in the product is a major problem for producers as well as for consumers (Wu et al., 2015). Galactose is a carbohydrate not only found in dairy products as a subunit of lactose molecules but also in many environments such as cereals, fruits, legumes, nuts, meats, seeds, and vegetables. It is present as a free form or bound to other carbohydrate units in various glycosidic linkages, such as α -1,6, β -1,3 and β -1,4, and as a component of lipids and proteins in cells (for more informations, review (Acosta & Gross, 1995)). The ability of starter cultures to metabolize lactose and the resulting galactose is therefore a major aspect of starter selection.

Thus, whereas the metabolism of lactose is complex, the genetics is well known. The first LAB that was completely sequenced, *Lactococcus lactis* subsp. *lactis* IL1403 came out in 2001 (Bolotin et al., 2001). According to ncbi, there are more than 102000 genomes available online

in 2017 (www.ncbi.nlm.nih.gov/genome). The study of LAB sequenced bacteria showed that *Lactobacillales* present a diversity in genome size ranging from 1.8 Mbp for *Streptococcus* to 3.7 Mbp for *Carnobacterium* (Cailliez-Grimal et al., 2013; Cailliez-Grimal, Afzal, & Revol-Junelles, 2014). Some unindustrialized LAB have a high genetic diversity (Rahman et al., 2014, 2016) which suggest high industrial potential.

Genome sequencing and the use of microbial genomics are powerful tools for investigation of cellular processes (Koonin, Aravind, & Kondrashov, 2000; Wei et al., 2002). The sequenced and annotated bacterial genomes permit the prediction of certain important characteristics researched by industrials. Thanks to comparative genomics, it became easy to search for metabolic pathways and this opens the possibility to consider the use of genomics to rapidly screen for functions of interest in the starter culture industry. Indeed, *Lactobacillus helveticus* CNRZ32 had been discovered to possess homologs of genes involved in the proteolysis of casein found in milk (Broadbent, Hughes, Welker, Tompkins, & Steele, 2013). The research project consists in predicting the formation of metabolic compounds *via* the exploration of the genomic content of CDS (Coding DNA Sequences) in comparison to previously annotated genomes.

Although the performance of automatic annotation is constantly improving, functional and relational annotations of genes still require human expertise. This is exemplified with the annotation of genes associated to metabolism, where the pathways are highly interconnected. Therefore, the annotation of metabolic pathways encoding genes requires a thorough dissection of the literature and requires a very good knowledge of the metabolic pathways and of the level of genetic variability that LAB can exhibit.

The aim of this review is to give an overview of the available data about lactose and galactose metabolism in order to facilitate expert annotation with a special focus on LAB. To do so, the literature was reviewed as well as the data available in 237 accessible genomes (complete or draft, see Table S1 for accession numbers) of LAB and some Gram-positive bacteria of the low GC subdivision, like *Bacillus*, *Listeria* and *Staphylococcus* (Axelsson, 2004). Genome analyses were mainly based on sequence alignment and synteny analyses which were performed by using the MicroScope platform as previously described (Iskandar et al., 2016; Vallenet et al., 2013).

2. Overview of lactose and galactose metabolism

After internalization into the cell, galactose or lactose can be metabolized thanks to two pathways: Leloir and Tagatose-6-Phosphate (Tagatose-6P) pathways (Figure 1) (Kandler, 1983). The end-product of the Leloir pathway is Glucose-1P, whereas the Tagatose-6P end products are triose-3 phosphates (Glyceraldehyde-3Phosphate (GPDH) and Dihydroxyacetone Phosphate (DHAP)). All these end products can subsequently enter the glycolysis pathway.

The canonical Leloir pathway is encoded by *galPMKTE*. Galactose and lactose are internalized by *galP*. Lactose is then hydrolyzed *via* a β -galactosidase (*lacZ* or *lacLM*) into glucose and β -galactose (Maxwell, Kurahashi, & Kalckar, 1962; Poolman, Royer, Mainzer, & Schmidt, 1989; van Rooijen, van Schalkwijk, & de Vos, 1991). The resulting galactose is subsequently metabolized into glucose-1P by the action of *galM* (galactose mutarotase), *galK* (galactose kinase) and *galT/galE* (galactose-1-phosphate uridylyltransferase, UDP-glucose-4-epimerase). Glucose-1P is then transformed into glucose-6P, an intermediate of the glycolysis, by the action of the phosphoglucumutase (encoded by *pgm*) (Figure 1).

In the canonical Tagatose-6P pathway, lactose-6P is hydrolyzed by a phospho- β -galactosidase (encoded by *lacG*) into glucose and galactose-6P. This latter is then transformed into 2 trioses (GPDH and DHAP) *via* a series of 3 reactions encoded by *lacAB* (galactose-6-phosphate isomerase), *lacC* (tagatose-6-phosphate kinase) and *lacD* (tagatose-1,6-diphosphate aldolase) (Figure 1).

Not all LAB are able to metabolize galactose. Indeed, although some strains of *Streptococcus thermophilus* are described to exhibit an active Leloir pathway (Anbukkarasi et al., 2014), strains of this species are generally known to partially ferment lactose. Thus, following lactose cleavage by the β -galactosidase encoded by *lacLM*, galactose negative *S. thermophilus* strains metabolize the glucose moiety, and excrete galactose in the medium thanks to the antiport permease *lacS*, which allows lactose/galactose exchange (Hutkins, Morris, & McKay, 1985; Poolman, 1993) (Figure 1). Similarly, *Lactobacillus delbruckii* ATCC 11482 and BAA-365 strains carry putative *galP*, *lacLM* putative genes (Table 1), and metabolize the glucose and release galactose in the extracellular medium (Crow & Thomas, 1984; Kafsi et al., 2014; Leong-Morgenthaler, Zwahlen, & Hottinger, 1991).

3. Detailed view of lactose and galactose metabolism

3.1. Transport

Lactose and galactose can enter the cells *via* either permeases encoded by *galP* or phosphotransferases systems encoded by *lacFE* (Figure 1). In general, permeases are non-specific transport systems (Forrest, Krämer, & Ziegler, 2011; Yan, 2013) except for *lacS* (lactose permease) found in *Streptococcus thermophilus* (Poolman et al., 1989).

In the Leloir pathway, the galactose can enter the cell *via* the permease encoded by *galP*, and the lactose *via* a permease encoded by *galP* or *lacS* (Fortina, Ricci, Mora, Guglielmetti, & Manachini, 2003; Leong-Morgenthaler et al., 1991; Neves et al., 2010). However, a putative *galP* gene is not always present in all genomes possessing the Leloir pathway (Table 1). However, the absence of the gene *galP* does not necessarily imply a deficiency in lactose/galactose use. Indeed, *Lactococcus lactis* NZ9000 inactivated in the gene *galP* internalizes galactose *via* an unknown PTS system. The resulting intracellular galactose-6P is subsequently dephosphorylated thanks to an unknown phosphatase, and the produced galactose enters the Leloir pathway (Neves et al., 2010) (Figure 1). Similarly, the genome of *Streptococcus mutans* (holding Leloir and Tagatose-6P pathways) lacks *galP* responsible for galactose uptake (Zeng, Das, & Burne, 2010). Instead, it would encode 14 putative PTS systems, of which one is specific for lactose (Ajdic & Ferretti, 1998). Although there is no PTS system specific for galactose in *S. mutans*, some data suggest that it can be transported by the lactose PTS with low affinity (Abranches, Chen, & Burne, 2004; Zeng et al., 2010).

On the other hand, in the Tagatose-6P pathway a PTS transporter, encoded by the genes *lacFE*, transports lactose and galactose. By contrast with the permease transport system, internalization through the PTS system leads to the phosphorylation of lactose and galactose (Abranches et al., 2004; Zeng et al., 2010; Zeng, Martino, & Burne, 2012). The PTS system is mainly encoded by *lacFE*, which can be duplicated in some cases (Table 1). It can be noticed that some strains would not contain *lacFE* homologues, as exemplified by *Pediococcus acidilactici* DSM20284, *S. agalactiae* A909 and *S. agalactiae* 2603 V/R (Table 1). It can be hypothesized that for these strains lactose or galactose may enter the cell through other PTS systems. Indeed, in general PTS are described as specific for one given carbohydrate. However, in some cases one PTS system can have the ability to transport another carbohydrate but with

lower affinity (Solopova et al., 2012). Thus, in *Streptococcus gordonii* DL1 galactose can enter the cell *via* PTS^{Lac}, PTS^{Man} or *via* PTS^{Gal} (Zeng et al., 2012).

It is generally accepted that internalisation of galactose through one transport system orientate the carbon flux towards one specific pathway. Thus, galactose internalization thanks to a PTS system, which lead to galactose phosphorylation, is linked to the Tagatose-6P pathway, whereas galactose internalization through a permease system, is linked to the Leloir pathway.

3.2. Beta-galactosidases

Lactose or phosphorylated lactose, depending on which transporter internalized lactose into the cell, prior catabolism through the Leloir or the Tagatose-6P pathway, require hydrolysis by the action of a β -galactosidase or phospho β -galactosidase, respectively (Figure 1). In the Leloir and Tagatose-6P pathways, these enzymes are encoded by *lacZ/lacLM* and *lacG*, respectively (Premi, Sandine, & Elliker, 1972). These two hydrolases belong to the large family of glycosyl hydrolases. Glycosyl hydrolases are divided into 45 families with 52 E.C. entries, according to an amino acid sequence comparison of 482 sequences (Henrissat, 1991; Henrissat & Bairoch, 1993). One given bacterium may contain several types of glycosyl hydrolases allowing to utilize different types of carbohydrates. Four of these 45 families (1, 2, 35 and 42) contain enzymes with β -galactosidase activity. The P- β -galactosidase *lacG* is a family 1 member, and the β -galactosidase *lacZ* is a family 2 member. It can be noticed that β -galactosidase can be produced extracellularly. Indeed, in *S. pneumoniae*, BgaA (β -1,4-galactosidase) and BgaC (β -1,3-galactosidase) are cell surface enzymes. They catalyze the hydrolysis of extracellular galactose-containing glycans, allowing to release free galactose which can subsequently enter the cell for catabolism *via* the Tagatose-6P or the Leloir pathways (Jeong et al., 2009; Paixão et al., 2015; Zähler & Hakenbeck, 2000) (Figure 1, Table 1).

Putative *lacG* genes are found in all genomes possessing the Tagatose-6P pathway (Table 1). Accordingly, the inactivation of *lacG* gene in *S. gordonii* DL1 leads to the inability of the strain to use lactose, whereas it is able to grow with galactose as carbohydrate source (Zeng et al., 2012). By contrast, putative *lacZ/lacLM* genes are not always found in all genome possessing the Leloir pathway suggesting the use of this pathway for galactose metabolism (Table 1). Indeed, inactivation of *lacZ/lacLM* in *Streptococcus salivarius* 57.I leads to the inability of the strain to use lactose while galactose can be metabolized, indicating that intracellular β -galactosidase is

essential for lactose metabolism (Chen, Betzenhauser, Snyder, & Burne, 2002). Among the 146 strains carrying putative Leloir pathway genes alone or in combination with the Tagatose-6P pathway genes, less than 40 strains possess homologue of *LacZ/LM* of *S. thermophilus*. Even if in these strains other β -galactosidases encoding genes can also be found in the vicinity of the Leloir pathway encoding genes, it does not imply that they are able to use lactose as substrate. Indeed, in *C maltaromaticum* 3.18, *Carnobacterium* sp. 17.4 and AT7 and *Enterococcus casseliflavus* ATCC12755 putative genes encoding β -galactosidases (*bgaB* and *bgaC*) are clustered with the genes putatively encoding the Leloir pathways (Figure 2A). These β -galactosidases would not be able to hydrolyse lactose, instead they would use other galactose-containing carbohydrate polymers (Coombs & Brenchley, 1999, 2001).

3.3.Leloir pathway

3.3.1. *galM*

Almost all genomes studied that possess the Leloir pathway genes, present alone or in combination with the Tagatose- 6P pathway genes, carry one putative *galM* gene (Table 1). The gene *galM* encodes a galactose mutarotase, which is not essential because its mutation does not affect the growth rate. It promotes the isomerization of β -galactose into α -galactose and the isomerization can take place spontaneously (Neves et al., 2010). It can be noticed that *galM* is present in strains without other *gal* and *lac* genes.

3.3.2. *galK*

The galactokinase encoded by *galK* phosphorylates α -D-galactose resulting in galactose-1P. Putative *galK* gene is essential and is found in all genome holding the Leloir pathway, and not present in all genome without this pathway (Table 1). Indeed, the deletion of *galK* impairs severely the ability of the strain *S. mutans* UA159 to metabolize lactose and galactose (Abranches et al., 2004).

3.3.3. *galT*

The galactose-1-phosphate uridylyltransferase encoded by *galT* convert galactose-1P to glucose-1P. The gene *galT* is essential and is found in all genome holding the Leloir pathway, and not present in all genome without this pathway (Table 1). Indeed, a *Lactococcus lactis*

NCD02054 *galT* mutant accumulates galactose-1P, which leads to the inability to grow on lactose and galactose (Vaughan, Pridmore, & Mollet, 1998).

3.3.4. *galE*

UDP-galactose 4-epimerase (*galE*) plays a role in the last step of the Leloir pathway. It is an essential enzyme and homologues are present in all genomes predicted to encode the Leloir pathway (Table 1). However, it can be present in bacteria without the Leloir pathway as well, indeed this enzyme is also involved in other metabolic functions such as exopolysaccharide synthesis (Grossiord, Luesink, Vaughan, Arnaud, & de Vos, 2003) (Figure 1).

3.3.5. Regulation of the Leloir pathway genes

The regulation gene *galR* encodes an activator of the *galKTE* and *lacSZLM* operons, and a negative regulator of its own expression. The gene *galR* is generally present in genomes holding the Leloir pathway (Table 1), except for *Bacillus subtilis* 168 and *S. gordonii* CH1 which are unable to utilize lactose and galactose *via* the Leloir pathways (Krispin & Allmansberger, 1998; Zeng et al., 2012). However, the presence an intact copy the gene *galR* does not necessarily lead to a galactose fermenting phenotype. Indeed, while some strains of *S. thermophilus* possess all the genes necessary to build an active Leloir pathway, they are unable to ferment galactose. These genes are silent thanks to the presence of a nucleotide substitution in the *galK* or *galR* promoter region (Anbukkarasi et al., 2014; Vaughan, van den Bogaard, Catzeddu, Kuipers, & de Vos, 2001).

The genes encoding the Leloir pathway are subjected to catabolic repression and are regulated by CcpA which binds to catabolic-responsive elements (*cre*) sites in the promoter region of the Leloir genes (Carvalho, Kloosterman, Kuipers, & Neves, 2011).

3.4. Tagatose-6-phosphate pathway

3.4.1. *lacAB*

Galactose-6P isomerase encoded by *lacAB* transforms galactose-6P into tagatose-6P. This enzyme is essential since the inactivation of these genes leads to the inability to grow on lactose and galactose containing medium and leads to the accumulation of galactose-6P (Abranches et al., 2004; Zeng et al., 2010, 2012). Therefore, it can be speculated that genomes without *lacAB* homologue does not have an active Tagatose-6P pathway (Table 1).

250 **3.4.2. *lacC***

251 The tagatose-6-phosphate kinase, encoded by *lacC*, phosphorylates the tagatose-6P in
252 order to form tagatose-1,6diP. It is generally present in the genomes possessing the other genes
253 encoding the Tagatose-6P pathway except in *Lactobacillus johnsonii* FI9785, *Lactobacillus*
254 *murinus* ASF361 and *Pediococcus acidilactici* DSM20284 (Table 1). Inactivation of *lacC* does
255 not impair growth in medium containing lactose and/or galactose as a source of carbohydrate
256 (Abranches et al., 2004; Zeng et al., 2010, 2012). *LacC* belongs to the pfkB family of
257 carbohydrate kinases, which includes ribokinase (RbsK) and 1-phosphofructokinase (FruK)
258 (Bork, Sander, & Valencia, 1993). It may present an enzymatic activity on fructose-6P when it is
259 present in the medium (Bissett & Anderson, 1980). This might explain the presence of *lacC* in
260 several copies in genomes predicted to encode the Tagatose-6P pathway as well as in five other
261 genomes where an incomplete Tagatose-6P pathway was predicted.

262 **3.4.3. *lacD***

263 The tagatose 1,6-diphosphate aldolase, encoded by *lacD*, is essential and promotes the
264 cleavage of Tagatose-1,6diP into 2 trioses. The *lacD* mutation causes the accumulation of
265 Tagatose-1,6diP in the medium, which leads to the inhibition of growth of the cells (Abranches
266 et al., 2004; Zeng et al., 2010, 2012). Importantly, the tagatose 1,6 diP aldolase may play a role
267 in the cleavage of Fructose-1,6diP (Bissett & Anderson, 1980) which could explain the fact that
268 *lacD* can be found in genomes where an incomplete Tagatose-6P pathway was predicted (Table
269 1).

270 **3.4.4. Regulation of the Tagatose 6-P**

271 The gene *lacR* is present in all genomes possessing the Tagatose-6P pathway and
272 encode a transcriptional repressor that binds the promoter region upstream *lacA*. It was
273 suggested that its repression activity is alleviated when galactose-6P and tagatose-6P binds LacR
274 (Oskouian & Stewart, 1990; van Rooijen, Dechering, Niek, Wilmink, & de Vos, 1993; Zeng et
275 al., 2010).

276 The Tagatose-6P genes are also regulated by *lacT*, which is present in some strains
277 holding the Tagatose-6P pathway, and encodes a transcriptional antiterminator protein present
278 within the *lac* operon of many LAB, that may play a role in the regulation down-stream *lacFEG*

expression (Richards, Choi, Bitar, Gurjar, & Stanhope, 2013; Zeng et al., 2012). By binding the mRNA transcript, LacT prevent the formation of a terminator and thus allows the RNA polymerase to transcribe the downstream genes (Declerck, Vincent, Hoh, Aymerich, & van Tilbeurgh, 1999). The antitermination activity of LacT is modulated by PTS dependant phosphorylation : when lactose or galactose PTS permease are engaged in sugar transport, LacT would be dephosphorylated, and would exert its antitermination activity (van Tilbeurgh & Declerck, 2001).

4. Distribution of the Leloir and the Tagatose-6P pathways at the genus and species levels

The contribution of the Leloir and the Tagatose-6P pathways to lactose and galactose metabolism was investigated at the genus and at the species levels (Table 1, Figure 3 A-B). In this analysis, each genus was represented by at least 3 species, and each species was represented by at least 8 strains. A genome is considered as a Leloir or Tagatose-6P pathway holder, if it possesses at least all the essential genes of one or the other pathway.

The analysis of presence and number of putative genes from Leloir (*gal* genes) or Tagatose-6P (*lac* genes) pathways indicated that, among the 237 strains, 4 different groups can be defined (Table 1): strains holding only the Leloir pathway genes (31%), strains holding only Tagatose-6P pathway genes (14%), strains holding both pathways genes (31%) and strains holding no pathway (24%).

Putative *gal* genes are presents in 72 strains holding the Leloir pathway alone and in 74 strains possessing Leloir and Tagatose-6P pathways (Table 1). Some strains can exhibit a partial Tagatose-6P pathway as exemplified by *C. maltaromaticum* 3.18 which lack a *lacB* homologue. It can be noticed that these *lac* homologues are dispersed in the genome and are likely involved in other cellular processes than lactose and galactose metabolism (Iskandar et al., 2016).

The proportion of bacteria predicted to have the Leloir pathway and/or the Tagatose-6P pathways is very high, of at least 80%. This is contrasting with *Listeria* where 15% of the genomes analyzed within this genus were predicted to encode a functional Leloir pathway and none were predicted to encode a functional Tagatose-6P pathway. This is in agreement with the idea that galactose and lactose metabolism is an important component of the ecology of these

bacteria. However, a high heterogeneity was recorded within each genome. For instance, the *Lactobacillus* genus contains bacteria that would exhibit either the Leloir pathway without the Tagatose-6P, the Leloir pathway in combination with the Tagatose-6P pathway, or none of these two pathways.

The Leloir pathway is in general highly represented within a given genus. On the other hand, the Tagatose-6P pathway is less well represented since it was not found in the genera *Leuconostoc*, *Listeria*, and *Weissella*. The concomitant presence of Leloir and Tagatose-6P pathways is present in 4 different genus, *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus*. The Tagatose-6P pathway is less well represented without the genes encoding the Leloir pathway as it is indeed represented in approximately 20% of *Carnobacterium*, *Lactococcus*, and *Streptococcus* genomes that do not exhibit a functional Leloir pathway (Figure 3.A). At the species level, the genetic profiles are rather homogenous (Figure 3.B).

Approximately half (5/9) of the species studied present only one profile, *i.e.* all the strains of the 2 species *Enterococcus faecalis* and *Streptococcus suis* are predicted to encode both pathways, all the strains of the 2 species *Staphylococcus aureus* and *S. pyogenes* would encode the Tagatose-6P pathway and not the Leloir pathway, and all the strains of the species *Listeria monocytogenes* would exhibit both pathways. One third (3/9) of the species show 2 different profiles (*Enterococcus faecium*, *Lactobacillus rhamnosus*, *Streptococcus sanguinis*), some strains of the same species possessing only Leloir pathway and the other the two pathways together. *Carnobacterium maltaromaticum* is the species exhibiting the highest intraspecific variability: some strains would possess the Leloir pathway, some others the Tagatose-6P pathway, one strain hold the *lac* and *gal* genes and some none of these pathways (Figure 3-B). This high intraspecific variability is in agreement with the high genetic diversity observed within the population of this species by MLST analysis (Rahman et al., 2014).

5. Organization of *lac* and *gal* genes

Lactose and galactose utilization by LAB revealed various combinations of the *gal* and *lac* genes (Breidt, Hengstenberg, Finkeldei, & Stewart, 1987; Chassy & Thompson, 1983; Grossiord, Vaughan, Luesink, & de Vos, 1998; Iskandar et al., 2016; Loughman & Caparon, 2007; Rosey & Stewart, 1992). While the *gal* genes are usually located on the chromosome, *lac*

genes of the Tagatose-6P pathway may be plasmidic or chromosomal. Although genes present high homology and conserved genomic synteny, the co-localizations are not always the same. The figures 2 A-B-C show the possible arrangements between the different genera and species.

6. Function of Leloir and Tagatose-6-P associated genes

Diversity within genomes can be relied to the functionality of each pathway in the cell. In fact, these two pathways play different roles in lactose and galactose metabolism.

The catabolysis of lactose and galactose *via* the Leloir pathway is described for *Lactococcus garviae* ATCC 49156, *L. lactis* IL 1403, *L. lactis* SK11, *L. lactis* MG1363 (Fortina, Ricci, & Borgo, 2009), *L. lactis* ATCC7962 (Vaughan et al., 1998), *Lactobacillus helveticus* ATCC 15009^T (Fortina et al., 2003), *S. thermophilus* (Anbukkarasi et al., 2014) (Table 1). By contrast, in strains of *Lactobacillus casei* (Bettenbrock, Siebers, Ehrenreich, & Alpert, 1999), *Lactobacillus rhamnosus* (Tsai & Lin, 2006), *L. lactis* (Siezen et al., 2005), *S. mutans* (Jagusztyn-Krynicka et al., 1992) (Table 1) lactose and galactose are catabolysed thanks to a Tagatose-6P pathway encoded by one *lac* gene cluster. In the strain *S. gordonii* CH1, the *lac* cluster is present in two copies, each cluster being specialized in the metabolism of one given carbohydrate: one cluster allows lactose utilization whereas the other permits the galactose metabolism (Zeng et al., 2010).

In some cases, *lac* and *gal* genes are present in the genome, like in *S. mutans*. It is believed that the expression of the Tagatose-6P pathway in *S. mutans* is inducible by lactose (Hamilton & Lebttag, 1979; Hamilton & Lo, 1978), whereas the expression of the Leloir pathway can be induced by galactose in the medium or by intracellular galactose released from polymers, such as melibiose (Ajdić, Sutcliffe, Russell, & Ferretti, 1996). However, this example cannot be generalized to all genomes holding *lac* and *gal* genes. In *S. gordonii* CH1, *gal* genes are present along with the *lac* genes, but only the *lac* genes are functional. It was hypothesized that the Leloir pathway is either not sufficiently active or is crossregulated by the *lac* genes (Zeng et al., 2012).

The functional divergence between copies of the *lac* genes can be dramatically more pronounced. One example is *S. pyogenes* which exhibits two *lac* gene clusters : Lac.1 and Lac.2 (Ferretti et al., 2001). Lac. 2 is dedicated to carbohydrate utilization and exhibit a canonical

genetic organization while Lac.1 deviated from this structure during evolution. The Lac.1 cluster is devoided of *lacG*, and the kinase encoding gene *lacC.1* is truncated. The gene *lacD.1* is a functional gene and predicted to encode a tagatose-1,6-bisphosphate aldolase. However, even if LacD.1 retains a aldolase activity *in vitro*, LacD.1 regulates virulence genes *in vivo* (Loughman & Caparon, 2006, 2007).

The Leloir pathway can be associated to anabolism in addition to carbohydrate catabolism. Indeed, the *galTE* product produces UDP-galactose when it converts galactose-1P to glucose-1P. The UDP-galactose can be subsequently used for exopolysaccharide synthesis (Boels, Ramos, Kleerebezem, & De Vos, 2001; Boels, van Kranenburg, Hugenholtz, Kleerebezem, & de Vos, 2001; Harutoshi, 2013).

Acknowledgments

The authors are grateful to the Genoscope, the MicroScope team, and the National Infrastructure “France Génomique”.

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Figure and Table legends

Figure 1: Schematic representation of the lactose and galactose metabolic pathways (according to (Neves et al., 2010; Price, Zeyniyev, Kuipers, & Kok, 2012; Zeng et al., 2010)). Enzymes encoded by the genes: *lacAB*, galactose-6-phosphate isomerase; *lacC*, tagatose-6-phosphate kinase; *lacD*, tagatose-1,6-diphosphate aldolase; *lacEF*, EIIABC^{lac/Gal}; *lacG*, phospho- β -galactosidase; *lacR*, transcriptional regulator; *lacS*, lactose permease; *lacZ*, β -galactosidase; *lacLM*, β -galactosidase; *galP*, galactose permease; *galM*, galactose epimerase; *galK*, galactose kinase; *galT*, galactose-1-phosphate uridyltransferase; *galE*, UDP-glucose-4-epimerase; *pgm*, phosphoglucomutase; *glk*, glucokinase. GPDH: glyceraldehyde 3 phosphate, DHAP: dihydroxyacetone phosphate. The dotted line symbolizes several reactions.

Figure 2: Organization and synteny between *gal* genes from the Leloir pathway and *lac* genes from the Tagatose-6P pathway among representative strains. A. *gal* genes from the Leloir pathway; B. *gal* and *lac* genes; C. *lac* genes from the Tagatose-6P pathway. The arrows show the gene direction. *galR* (regulation gene), *galK* (galactose kinase), *galM* (galactose mutarotase), *galT* (Galactose-1P uridyltransferase), *galE* (UDP-glucose 4-epimerase), *lacZ/lacLM* (beta galactosidase), *galP/lacS* (galactose/lactose permease), *agaR* (alpha-galactosidase), *bgaB* (beta-galactosidase), *bgaC* (beta-galactosidase), *bglH* (beta-glucosidase). *lacE* (PTS system), *lacF* (PTS system), *HP* (hypotetical protein), *araC* (AraC family transcriptional regulator), *pgcA* (alpha-phosphoglucomutase), *ganB* (arabinogalactan endo-1,4-beta-galactosidase), *α -gal* (alpha/beta hydrolase family), *msm* (multiple sugar metabolism), *lacX* (putative galactose mutarotase, plasmid protein). *lacAB* (galactose isomerase), *lacC* (Tagatose6P kinase), *lacD* (tagatose-1,6P aldolase), *lacFE* (PTS system), *lacG* (P-beta galactosidase), *lacR* (regulation genes), *trp* (transposase), *lacT* (anti-terminator transcriptional gene).

Figure 3: Diversity of the lactose and galactose metabolic pathways within (A) genus and (B) species of the different genus analyzed. The data used to build this graph were obtained from the genomes hosted in the MicroScope platform and from the results described in a previous study (Iskandar et al., 2016). The numbers between brackets on the X axis indicate the total number of strains analyzed in each genus.

Leloir Pathway
 Tagatose-6P Pathway
 Leloir and Tagatose -6P Pathways
 No Pathway

676 **Table 1 : Lactose/galactose metabolism genes present in LAB and non-LAB strains.**

677 The shadowed boxes indicate the presence of homologue: light gray indicates that only one copy
 678 of the gene is present and the dark gray indicates that more than one copy are present. The
 679 numbers indicate the number of copies of paralogues. *lacAB* (galactose isomerase), *lacC*
 680 (tagatose-6P kinase), *lacD* (tagatose-1,6P aldolase), *lacFE* (PTS system), *lacG* (P-β
 681 galactosidase), *lacR* (regulation genes), *lacS* (lactose permease), *lacLMZ* (βgalactosidase), *galP*
 682 (galactose permease), *galM* (galactose epimerase), *galK* (galactose kinase), *galT* (galactose-1P-
 683 uridyltransferase), *galE* (UDP-glucose-4-epimerase).

Figure 1: Schematic representation of the lactose and galactose metabolic pathways (according to Neves et al., 2010; Price et al., 2012; Zeng et al., 2010). Enzymes encoded by the genes: *lacAB*, galactose-6-phosphate isomerase; *lacC*, tagatose-6-phosphate kinase; *lacD*, tagatose-1,6-diphosphate aldolase; *lacEF*, EIIABC^{lac/Gal}; *lacG*, phospho-β-galactosidase; *lacR*, transcriptional regulator; *lacS*, lactose permease; *lacZ*, β-galactosidase; *lacLM*, β-galactosidase; *galP*, galactose permease; *galM*, galactose epimerase; *galK*, galactose kinase; *galT*, galactose-1-phosphate uridyltransferase; *galE*, UDP-glucose-4-epimerase; *pgm*, phosphoglucomutase; *glk*, glucokinase. GPDH: glyceraldehyde 3 phosphate, DHAP: dihydroxyacetone phosphate. The dotted line symbolizes several reactions.

Figure 1

Figure 2: Organization and synteny between *gal* genes from the Leloir pathway and *lac* genes from the Tagatose-6P pathway among representative strains. A. *gal* genes from the Leloir pathway; B. *gal* and *lac* genes ; C. *lac* genes from the Tagatose-6P pathway. The arrows show the gene direction. *galR* (regulation gene), *galK* (galactose kinase), *galM* (galactose mutarotase), *galT* (Galactose-1P uridyltransferase), *galE* (UDP-glucose 4-epimerase), *lacZ/lacLM* (beta galactosidase), *galP /lacS* (galactose/lactose permease), *agaR* (alpha-galactosidase), *bgaB* (beta-galactosidase), *bgaC* (beta-galactosidase), *bglH* (beta-glucosidase). *lacE* (PTS system), *lacF* (PTS system), *HP* (hypotetical protein), *araC* (AraC family transcriptional regulator), *pgcA* (alpha-phosphoglucomutase), *ganB* (arabinogalactan endo-1,4-beta-galactosidase), *α-gal* (alpha/beta hydrolase family), *msm* (multiple sugar metabolism), *lacX* (putative galactose mutarotase, plasmid protein). *lacAB* (galactose isomerase), *lacC* (Tagatose6P kinase), *lacD* (tagatose-1,6P aldolase), *lacFE* (PTS system), *lacG* (P-beta galactosidase), *lacR* (regulation genes), *trp* (transposase), *lacT* (anti-terminator transcriptional gene).

Figure 2A

Figure 2A (continued)

Leuconostoc citreum KM20

L. gasicomitatum LMG 18811

L. gelidum JB7

L. kimchii IMSNU11154

L. mesenteroides ssp. *mesenteroides* J18, ATCC8293

L. mesenteroides ssp. *mesenteroides* ATCC8293

Listeria grayi DSM20601

Oenococcus oeni AWRIB429, ATCC BAA-1163, PSU-1

Pediococcus acidilactici DSM 20284

P. pentosaceus ATCC 25745

Streptococcus sanguinis SK353

S. salivarius SK126

Streptococcus thermophilus LMG18311

S. salivarius CCHSS3

Weissella confusa LBAE C39-2

Weissella cibaria KACC 11862

W. paramesenteroides ATCC 33313

Weissella koreensis KACC 15510, KCTC 3621

Figure 2B

Enterococcus avium ATCC14025

Enterococcus faecalis TX1322

Enterococcus faecalis V583

Lactobacillus rhamnosus CASL

Lactobacillus rhamnosus 21052

Lactobacillus casei BL23

L. casei ATCC334 (chr)

L. casei ATCC334 (pl.)

L. curvatus CRL705

Lactococcus lactis SK11 (chr)

L. Lactis SK11 (pl.)

Streptococcus agalactiae A909

Streptococcus infantarius ATCC BA-102

Streptococcus gordonii CH1

Streptococcus mutans UA159

Figure 2C

Figure 3: Diversity of the lactose and galactose metabolic pathways within (A) genus and (B) species of the different genus analyzed. The data used to build this graph were obtained from the genomes hosted in the MicroScope platform and from the results described in a previous study (Iskandar et al., 2016). **The numbers between brackets on the X axis indicate the total number of strains analyzed in each genus.**

Leloir Pathway, Tagatose-6P pathway, Leloir and Tagatose-6P Pathways, No Pathway

Figure 3-A

Figure 3-B

Table 1

	Tagatose-6Phosphate pathway									Leloir pathway						
	lacR	lacA	lacB	lacC	lacD	lacE	lacF	lacG	lacT	galP lacS lacY	lacZ lacLM	galR	galM	galK	galT	galE
Bacillus subtilis 168	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
Carnobacterium gilichinskyi WN1359	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Carnobacterium maltaromaticum 3.18	1	2	0	1	2	1	1	1	0	0	0	1	1	1	1	2
Carnobacterium sp. 17-4	0	0	0	2	1	0	0	0	0	1	0	1	1	1	1	2
Carnobacterium sp. AT7	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1
Enterococcus durans ATCC6056	0	0	0	1	1	0	0	0	0	1	0	1	1	1	1	1
Enterococcus faecium D344SRF, DO NZ_AAAK, TX0133a04, TX0133B, TX0082, E1071, E1636, U0317, E1679, DO NZ_AC1Y, TX0133a01, TX0133A, TX0133C, E1162, E1039,	0	0	0	0	0	1	1	1	0	1	0	1	1	1	1	1
Enterococcus hirae ATCC9790	0	0	0	1	1	0	0	0	0	1	0	1	1	1	1	1
Enterococcus mundtii QU25	0	0	0	0	2	1	1	1	0	1	0	1	1	1	1	1
Lactobacillus acidophilus NCFM	0	0	0	0	0	0	0	0	0	1	2	1	1	1	1	1
Lactobacillus brevis ATCC27305	0	0	0	0	0	0	0	0	0	2	1	1	1	1	1	1
Lactobacillus brevis ATCC14869, KB290	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
Lactobacillus brevis ATCC367	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
Lactobacillus fermentum IFO3956	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Lactobacillus helveticus DPC4571	0	0	0	0	0	0	0	0	0	1	2	1	1	1	1	1
Lactobacillus johnsonii NCC533	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	2
Lactobacillus plantarum ATCC14917	0	0	0	0	0	0	0	0	0	2	1	1	1	1	1	1
Lactobacillus plantarum JDM1, ST-III	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2
Lactobacillus plantarum WCFS	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2
Lactobacillus rhamnosus ATCC5462	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
Lactobacillus sakei 23K	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2
Lactobacillus salivarius ATCC11741, GJ-24, NIAS840	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2
Lactobacillus salivarius SMXD51, UCC118	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	2
Lactobacillus sp. ASF360	0	0	0	0	0	0	0	0	0	2	1	1	1	1	1	3
Lactococcus garvieae ATCC49156	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
Lactococcus lactis KF147	0	0	0	0	0	0	0	0	0	1	1	1	1	2	2	1
Lactococcus lactis MG1363, IL403	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Lactococcus piscium MKFS47	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1	1
Leuconostoc citreum KM20	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Leuconostoc gasicomitatum LMG18811	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Leuconostoc gelidum JB7	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Leuconostoc kimchii IMSNU11154	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Leuconostoc mesenteroides J18	0	0	0	0	0	0	0	0	0	3	1	1	1	2	1	1
Leuconostoc mesenteroides ATCC8293	0	0	0	0	0	0	0	0	0	3	1	1	1	3	2	2
Listeria grayi DSM20601	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
Oenococcus oeni AWRIB429, ATCC BAA- 1163, PSU-1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
Pediococcus pentosaceus ATCC25745	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1

<i>Streptococcus salivarius</i> CCHSS3	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
<i>Streptococcus salivarius</i> SK126	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1
<i>Streptococcus sanguinis</i> SK353	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
<i>Streptococcus thermophilus</i> CNRZ1066, LMG18311, LMD-9	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2
<i>Weissella cibaria</i> KACC11862	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
<i>Weissella confusa</i> LBAE C39-2	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
<i>Weissella koreensis</i> KCTC3621, KACC15510	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
<i>Weissella paramesenteroides</i> ATCC33313	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1
<i>Enterococcus avium</i> ATCC14025	1	1	1	2	2	2	2	2	0	1	0	1	1	1	1	1
<i>Enterococcus casseliflavus</i> ATCC12755	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1
<i>Enterococcus faecalis</i> V583, TX0635, TX0411, HH22, DAPTO 512, DAPTO 516, R712, S613, TX0104, TX4248, TX1322, TX2134, TX0855, TX0860, TUSoD Ef11, TX0470, PC1.1, ATCC29200, TX0109, TX0102	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1
<i>Enterococcus faecium</i> E980, PC4.1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1
<i>Enterococcus faecium</i> TX1330	2	2	2	2	2	2	2	2	0	1	0	1	1	1	1	1
<i>Enterococcus italicus</i> DSM15952	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1
<i>Lactobacillus curvatus</i> CRL705	1	2	2	1	2	1	1	1	0	0	0	1	1	1	1	2
<i>Lactobacillus johnsonii</i> FI 9785	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1
<i>Lactobacillus murinus</i> ASF361	1	1	1	0	2	1	1	1	1	1	1	1	1	1	1	1
<i>Lactobacillus paracasei</i> 8700:2	1	1	1	2	2	2	1	1	0	1	0	1	1	1	1	1
<i>Lactobacillus rhamnosus</i> CASL, HN001, R0011, LMS2_1, ATCC21052, LC705	1	1	1	1	1	2	2	2	2	0	0	1	1	1	1	1
<i>Lactobacillus rhamnosus</i> GG ATCC53103	1	1	1	1	2	1	1	1	1	0	0	1	1	1	1	1
<i>Lactobacillus casei</i> ATCC334	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
<i>Lactobacillus casei</i> BL23	1	1	1	1	2	1	1	1	1	0	0	1	1	1	1	1
<i>Lactococcus lactis</i> SK11	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1
<i>Pediococcus acidilactici</i> DSM20284	1	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1
<i>Streptococcus agalactiae</i> A909	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	2
<i>Streptococcus gordonii</i> CH1	1	2	2	2	3	2	2	1	1	0	0	0	0	1	1	1
<i>Streptococcus infantarius</i> ATCC-BA-102	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	2
<i>Streptococcus mutans</i> UA159	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	2
<i>Streptococcus pneumoniae</i> D39	1	1	1	1	1	2	2	2	1	0	0	1	1	1	2	3
<i>Streptococcus pneumoniae</i> R6, TIGR4	1	1	1	1	1	1	1	1	1	0	0	1	1	1	2	3
<i>Streptococcus sanguinis</i> ATCC29667, ATCC49296, 2908, SK36, SK335, SK678, SK1087, SK1057	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
<i>Streptococcus sanguinis</i> SK1, SK1056, SK1058, SK1059, SK115, SK160, SK340, SK405, SK408, SK49, SK72, SK150, SK330	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	2
<i>Streptococcus sanguinis</i> VMC 66	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1
<i>Streptococcus suis</i> 05ZYH33, ST3, ST1, SS12, JS14, 98HAH33	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
<i>Aerococcus viridans</i> ATCC11563	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
<i>Carnobacterium maltaromaticum</i> ATCC35586	1	2	2	2	3	2	2	1	0	0	0	0	0	0	0	1
<i>Carnobacterium maltaromaticum</i> LMA28, DSM20342 MX5	1	2	2	2	1	2	2	2	0	0	0	0	0	0	0	0
<i>Lactococcus raffinolactis</i> 4877	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
<i>Staphylococcus aureus</i> 16K, JKD6008, MRSA252, TW20, Z172, T0131, S0385, COL, N315, USA300-TSACH1516, CA-347, USA300-FPR3757, Mu50, MW2, 08BA02176, JH1, 11819-97, Newman NCTC8325	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
<i>Streptococcus agalactiae</i> 2603 V/R	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	1
<i>Streptococcus agalactiae</i> NEM 316	2	2	2	2	2	1	1	1	1	0	0	0	0	0	0	1

[illegible]