

# Mannitol production in Lactococcus Lactis

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# Mannitol production in *Lactococcus Lactis*

PhD thesis

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# Summary

Mannitol is a six-carbon polyol with many applications in food and therapeutic area, due to its low glycemic index, low insulin response, low calorie, osmotic and anti-oxidative traits. Microbial mannitol production, particularly using Lactic acid bacteria (LAB) has been extensively studied and most of these have relied on reducing fructose via mannitol dehydrogenase (MDH). *Lactococcus lactis* (*L. lactis*), a homofermentative LAB with a long history of safe use in the food industry, is equipped with all the necessary enzymes for mannitol production by reducing fructose-6-phosphate (F6P), which means it could produce mannitol from various sugars, and is therefore considered as a new promising host for mannitol production. Nevertheless, the low expression level of the relevant genes for producing mannitol renders *L. lactis* a low efficient mannitol producer. This dilemma has been bypassed by introducing heterologous genes; however, such strains are not food-grade and are limited for their applications.

In this study, we found that *L. lactis* has an inherent capacity to produce mannitol from glucose. By adaptively evolving *L. lactis*, or derivatives blocked in $NAD^+$ regenerating pathways, we managed to accelerate growth on mannitol. When cells of such adapted strains are resuspended in buffer containing glucose, 4-58% of the glucose metabolized is converted into mannitol, in contrast to non-adapted strains. Whole genome sequencing of these mutants revealed one critical mutation, C-39T, in the promoter region of *mtlA* gene, which appear to enhance the promoter strength.

Bioinformatics analysis of the mannitol operon and reporter gene assay showed the expression of *mtlD* gene, encoding mannitol-1-phosphate dehydrogenase (M1PDH), which is a key enzyme for mannitol production, is tightly controlled by transcriptional regulator MtlR and CcpA together. In addition, we found that the expression of the mannitol operon could be enhanced by shifting cells to stationary phase and by deleting the *mtlF* gene.

Production of mannitol in *L. lactis* will indirectly cost ATP due to the phosphoenolpyruvate (PEP) consumption during glucose transportation, and indeed, increasing the energy supply by adding arginine to the growth medium or activating the acetate-producing pathway also resulted in a stimulation of mannitol production. By applying these combined findings, we developed a two-step fermentation setup, i.e., growing cells to stationary phase, then incubating them with limited aeration. Using this method and glucose as a substrate, we could achieve 6.1 g/L mannitol with 60% yield using a food-grade strain. 9.8 g/L mannitol and 57% yield was

obtained using an *mtlD*-overexpressed strain, which to our knowledge are the highest yield and titers reported to date, respectively. Furthermore, we showed that *L. lactis* could produce mannitol solely from fructose, galactose, or maltose, which demonstrates the great potential of using *L. lactis* as a mannitol producer.

Our studies have resulted in a deep insight into the mechanism for mannitol production in *L. lactis*, as well as provided clues to further develop a food-grade *L. lactis* that could efficiently produce mannitol from various sugars.

# Danske Resume

Mannitol er en seks-carbon polyol med mange anvendelser inden for mad og klinisk område på grund af dets lave glykæmiske indeks, lave insulinrespons, lave kalorieindhold, osmotiske og antioxidative egenskaber. Mannitolproduktion fra mikrober, især fra mælkesyrebakterier (LAB) er blevet grundigt undersøgt. Imidlertid er de fleste af resultaterne, der producerer mannitol, afhængige af at reducere fruktose med mannitol dehydrogenase (MDH). *Lactococcus lactis* (*L. lactis*), en homofermentativ LAB med en lang historie med sikker anvendelse i fødevareindustrien, udstyret med alle nødvendige enzymer til mannitolproduktion ved at reducere fruktose-6-phosphat (F6P), hvilket betyder, at det kunne producere mannitol fra forskellige sukker betragtes derfor som en ny lovende vært til produktion af mannitol. Ikke desto mindre gør det lave ekspressionsniveau for de relevante gener til produktion af mannitol *L. lactis* til en lav effektiv mannitolproducent. Dette dilemma kunne omgåes ved at indføre heterologe gener; sådanne stammer er imidlertid ikke fødevarekvalitet og er begrænset til deres anvendelse.

I denne undersøgelse finder vi, at *L. lactis* har en iboende evne til at danne mannitol fra glucose. Ved adaptivt at udvikle *L. lactis* eller derivater blokeret i $NAD^+$ regenereringsveje lykkes det os at fremskynde væksten på mannitol. Når celler i de tilpassede stammer resuspenderes i buffer indeholdende glucose, omdannes 4-58% af den metaboliserede glucose til mannitol i modsætning til ikke-tilpassede stammer. Genomsekventering af disse mutanter afslørede en kritisk mutation, C-39T, i promotorregionen af mtlA-genet, som kunne forbedre promotorstyrken. Bioinformatikanalyse af hele mannitoloperon- og reportergenassayet viste ekspressionen af *mtlD*-gen, der koder for mannitol-1-phosphatdehydrogenase (M1PDH), som er et nøgleenzym til produktion af mannitol, styres tæt af transkriptionsregulator MtlR og CcpA sammen. Desuden fandt vi, at ekspressionen af mannitoloperon også kunne forbedres ved at skifte celler til stationær fase og ved at slette *mtlF*-genet. Da produktion af mannitol i *L. lactis* indirekte vil koste ATP, resulterer energiforsyning ved tilsætning af arginin eller genvinding af acetatproducerende vej også i en positiv effekt på mannitolproduktionen. Ved at anvende disse fund udviklede vi en to-trins fermenteringsopsætning, dvs. voksende celler til stationær fase og derefter inkuberes med begrænset beluftning. Ved hjælp af denne metode, fra glucose, kunne vi opnå 6,1 g/L mannitol med 60% udbytte ved hjælp af en fødevarestamme og 9,8 g/L mannitol med 57% udbytte ved hjælp af en *mtlD*-overudtrykt stamme, som er det højeste udbytte titer har nogensinde rapporteret henholdsvis. Desuden viste vi, at *L. lactis* kun kunne

producere mannitol ud fra fruktose, galactose eller maltose, hvilket viser de store fordele ved at bruge *L. lactis* som en mannitolproducent.

Alt i alt giver vores undersøgelse en dyb indsigt i mekanismen til mannitolproduktion i *L. lactis* og giver understøttelser til yderligere udvikling af en fødevarekvalitet *L. lactis*, der effektivt kunne producere mannitol fra forskellige sukkerarter.

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May, 2021

# Outline of this thesis

**Chapter 1**

Background of the current study. Introduction of mannitol, *Lactococcus Lactis*, and microbial production of mannitol.

**Chapter 2**

Mannitol production in *L. lactis* can be enhanced by adapting cells in mannitol and by restricting NADH flux in cells.

This chapter is based on our work ‘‘Harnessing Adaptive Evolution to Achieve Superior Mannitol Production by *Lactococcus lactis* Using Its Native Metabolism’’, which has been published by *Journal of Agricultural and Food Chemistry*, 2020.

**Chapter 3**

Mannitol production can be stimulated by the expression of mannitol operon, which is tightly controlled by transcriptional regulator CcpA and MtlR. The expression of mannitol operon can be enhanced by inactivating *mtlF* gene or shifting cells into stationary phase.

This chapter is based on our work ‘‘Deciphering the regulation of the mannitol operon paves the way for efficient production of mannitol in *L. lactis*’’, which has been accepted by *Applied and Environmental and Food Microbiology*, 2021.

**Chapter 4**

Food-grade mannitol production by using stationary cells from different sugars.

This chapter is partially based on of our work ‘‘Harnessing Adaptive Evolution to Achieve Superior Mannitol Production by *Lactococcus lactis* Using Its Native Metabolism, *Journal of Agricultural and Food Chemistry*, 2020’’ and ‘‘Deciphering the regulation of the mannitol operon paves the way for efficient production of mannitol in *L. lactis, Applied and Environmental and Food Microbiology*, 2021’’.

**Appendix**

Supplementary tables, figures and data for supporting this study.

# Frequently occurred abbreviations

| | |
|---|---|
| LAB | Lactic acid bacteria |
| *L. lactis* | *Lactococcus lactis* |
| MDH | Mannitol dehydrogenase |
| F6P | Fructose-6-phosphate |
| M1P | Mannitol-1-phosphate |
| M1PDH | Mannitol-1-phosphate dehydrogenase |
| M1Pase | Mannitol-1-phosphatase |
| PEP | Phosphoenolpyruvate |
| PTS | Phosphotransferase system |
| ATP | Adenosine triphosphate |
| NADH | Nicotinamide adenine dinucleotide |
| EIIA | Phosphotransferase system enzyme IIA |
| CcpA | Catabolite control protein A |
| Hpr | Phosphocarrier protein |
| P~Ser46-Hpr | Serine 46 phosphorylated phosphocarrier protein |
| CCR | Carbon catabolic repression |

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# Chapter 1. Introduction

## 1.1 Lactic acid bacteria and their applications

Lactic acid bacteria (LAB) are defined as a group of Gram-positive organisms that mainly ferment sugars into lactic acid. Including *Streptococcus thermophilus* (*S. thermophilus*), *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus salivarius* (*L. salivarius*), and *Lactococcus lactis* (*L. lactis*), etc. Many of those are generally recognized as safe (GRAS) and have a long history of being used in the food industry and currently have been extended to produce chemicals, vitamins, and bioactive peptides, etc. Their high value of economical uses and critical role in our modern life increase the demand and interest for a better understanding of them.

### 1.1.1 Classification and Genomic features

#### Classification

LAB widely exist in nature, such as in dairy products, pickled vegetables, decomposing fruits, and cavities of humans and animals. LAB have been characterized as low GC, non-spore forming, catalase-negative, low-pH tolerance bacteria. Base on their cellular morphology, source of energy, growth temperature, and other specific properties, the classification of LAB was employed as *Streptococcus*, *Betacoccus*, *Tetracoccus*, *Thermobacterium*, *Streptobacterium*, *Betabacterium*, *Microbacterium*, and *Bacterium bifidum* were described by Orla Jensen in the 1920s (1).

However, such traditional classifications like this used for almost a century does not agree with the results from rRNA-based phylogeny. From the current taxonomy, combining the rRNA analysis with the applications in the fermentation of food and feed products, LAB generally contain the genera *Enterococcus*, *Lactococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Carnobacterium*, *Leuconstoc*, *Weissella*, and *Lactobacillus*, where the last one is the largest group comprising at least 50 species (2)(3)(4)(5). Additionally, LAB are also categorized into homofermentative and heterofermentative groups, according to their fermentative products. Homofermentative LAB mainly produce lactic acid whereas heterofermentative LAB additionally produce other metabolites such as ethanol, acetic acid, acetoin, and CO2 (4).

## Genomic features

Genome information of LAB was firstly released from *Lactococcus lactis spp. lactis* IL1403 in 2001 (6). With the rapid development of the sequencing techniques in the last two decades, more than 200 LAB strains have been sequenced (7)(8). The genome of LAB were characterized as low GC content and ranging from 1.23 Mb to 4.91 Mb (9). From comparative genomics studies, it showed all LAB possess a mutual *Lactobacillales* ancestor but experienced a great gene loss and metabolic simplification during their evolution (Figure 1.1), which was deduced to result from adaptation in the nutrients rich environments (10). Genetic events such as horizontal gene transfer, gene duplication, and genome reduction result in the present shape and structure of the LAB genome (11).

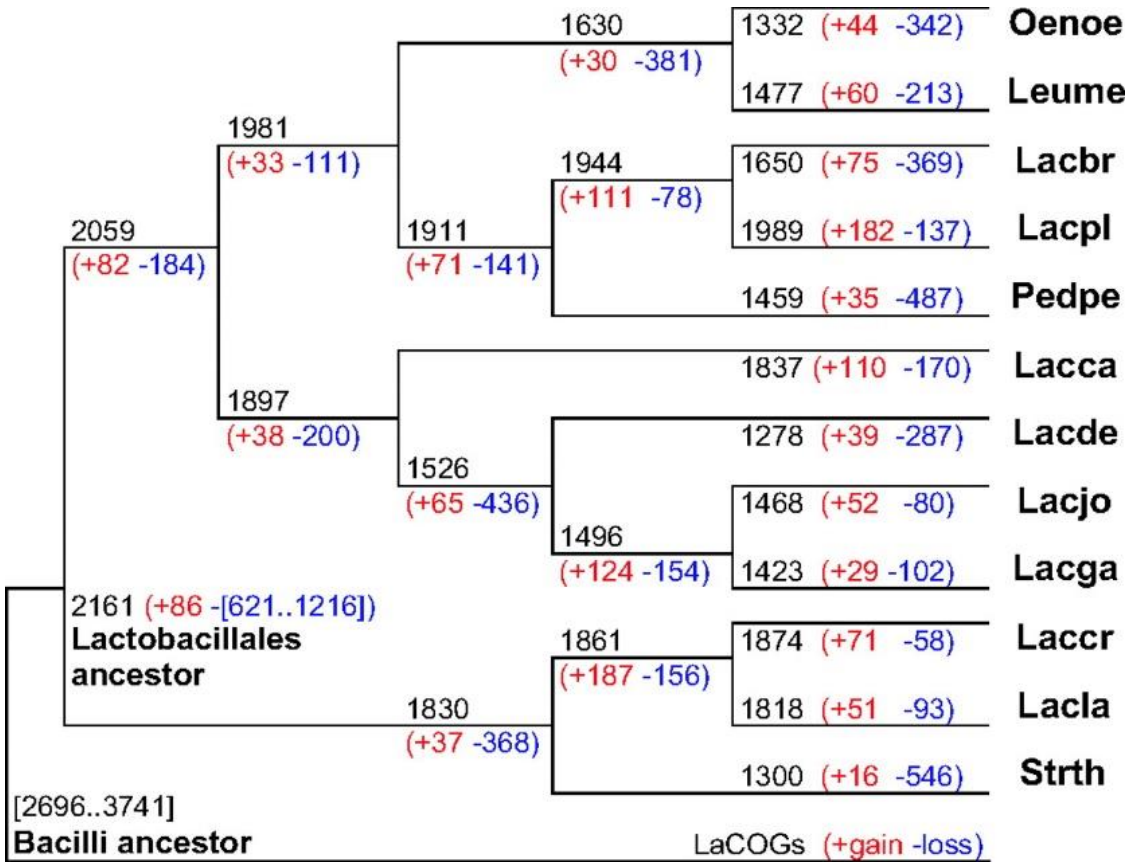


**Figure 1.1. Reconstruction of gene content evolution in *Lactobacillales*.** The tree topology is rooted by using *Bacillus subtilis* as the outgroup. For each species and each internal node of tree, the inferred number of LaCOGs present, and the numbers of LaCOGs lost (blue) and gained (red) along the branch leading to the given node (species) are indicated. *Lacga, Lb. gasseri; Lacbr, Lb. brevis; Pedpe, P. pentosaceus; Laccr, Lc. lactis ssp. cremoris; Strth, S. thermophilus; Oenoe, O. oeni; Leume, Le. mesenteroides; Lacca, Lb. casei; Lacde, Lb. delbrueckii; Lacla, Lc. lactis; Lacpl, Lb. plantarum; Lacjo, Lb. johnsonii.* Reprinted with permission from (10). Copyright (2006) National Academy of Sciences, U.S.A.

### 1.1.2 Genernal applications

Many LAB are versatile non-virulent bacteria that could be found in many of our food products. The primary application of LAB in the food industry is used as starter culture, involving fermenting cheese, milk, yogurt, meat, fish, fruit, cereals, and vegetables, and contributing to extra nutritional value, flavor, and texture to these fermented foods.

As the primary product from LAB, lactic acid could inhibit the growth of virulent bacteria, which would lead to a safer fermentation process and prolong the shelf life of products. In another hand, numerous health-promoting bioactive compounds could be released by LAB, which increases the nutrient value in fermented food products (12). It was showed fermented milk, vegetables, meats, cheese, etc., contain a large amount of live LAB at consumption and around 1% of them could survive after passing through the human gastrointestinal tract (13)(14). In fact, LAB could naturally be found in the human intestine though it is still hard to characterize the contribution of them (15). Considering LAB could resist the stomach and bile efflux, it is promising to develop live delivery vectors with the expression of therapeutic molecules by using LAB, and in fact, several achievements have been reported for using *Lactococcus, Lactobacillus,* and *Bifidobacterium* as delivery vectors (16). Furthermore, LAB are also well-known bacteriocin producers. Bacteriocin gives the producer an advantage over other bacteria in competing for nutrients in nature. Thus, bacteriocin could be applied in food reservation by used as inhibitors for food-spoiling bacteria and food-borne pathogens (17). By analyzing 238 complete LAB genomes, a recent study showed a total number of 785 putative bacteriocins, where 500 were from *Streptococcus,* 204 were from *Lactobacillus,* and 32 from *Lactococcus,* etc (18). Besides, LAB are excellent hosts for producing chemical compounds and vitamins, some of those contribute to flavor and high nutrient value in food products, or could be used in other areas such as synthesis of commodity chemicals, biofuels, or pharmaceuticals (19).

## 1.2 *Lactococcus lactis*

*Lactococcus lactis* (*L. lactis*), previously classified as streptococcus genus until 1985, has three subspecies, *L. lactis subsp. cremoris, L. lactis subsp. lactis and L. lactis subsp. hordniae* (20). *L. lactis* has been among the most used food-fermenting bacteria in the world for centuries. They are characterized as spherical, homolactate, facultative anaerobic, and to date, have been developed to hundreds of strains and biovariants due to their high industrial value (21)(22).

### 1.2.1 Genome

The plasmid-free strains *L. lactis ssp. lactis* IL1403 and *L. lactis ssp. cremoris* MG1363 are the most characterized species in *L. lactis*. IL1403 and MG1363 share around 85% identity of sequence similarity and deduced to have been diverged since 17 million years ago (23). Both IL1403 and MG1363 have been proved diploid (24). The ability for citrate utilization is the main difference for distinguishing MG1363 and IL1403, as MG1363 has no citrate lyase activity in cells (25). MG1363 was derivated from strain NCDO712 by eliminating its plasmids using UV treatment and protoplast strategies in 1983 (26). Genome sequencing showed the size of MG1363 genome is around 2.5 Mb with an average GC content of 35.8%, containing more than 2400 protein-coding genes and at least 10 different IS elements (27). The availability of genome data allows the possibility for further applied and fundamental research. Besides using in the food industry, now MG1363 has become a prototype strain for studying *L. lactis* and has been developed for various biotechnological applications, such as cell factories for producing chemicals or therapeutic proteins (20)(28).

### 1.2.2 Central Metabolism

The broad application of *L. lactis* makes it considered a promising target for metabolic engineering. The valuable compounds produced from *L. lactis*, such as lactic acid, ethanol, acetoin, and diacetyl, which could contribute to flavor, texture, or probiotic use in food application, are almost derived and converted from the absorbed sugars. Engineering and harnessing the sugar metabolism, i.e., the central metabolism is considered an efficient way to get a high yield of those products. Besides, central metabolism also involves ATP synthesis, redox balance, and metabolic modulation, which accounts for the main energy source and fundamental biochemical reactions for maintaining *L. lactis* life activities. Due to its important role, the central carbon metabolism in *L. lactis* has been well characterized by far (Figure 1.2). In general, cells normally gain 2 net ATP and 2 net NADH per glucose, which has been an important clue for engineering *L. lactis*. Furthermore, the activity of key glycolytic enzymes phosphofructokinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), fructose 1,6-bisphosphate aldolase (FBA), phosphofructokinase (PFK), and concentrations of key glycolytic intermediates 2-phosphoglycerate, phosphoenolpyruvate, 3-phosphoglycerate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate are well-characterized and shown to affect the speed and direction of glycolytic flux (29). The primary central metabolism in *L. lactis* is considered simple and therefore has been served as a target

for metabolic engineering in the last two decades. The understanding of the central metabolism in *L. lactis* makes people possible to develop strategies for rewiring the glycolytic flux and further to get excellent yield and titers of interested products from *L. lactis*.

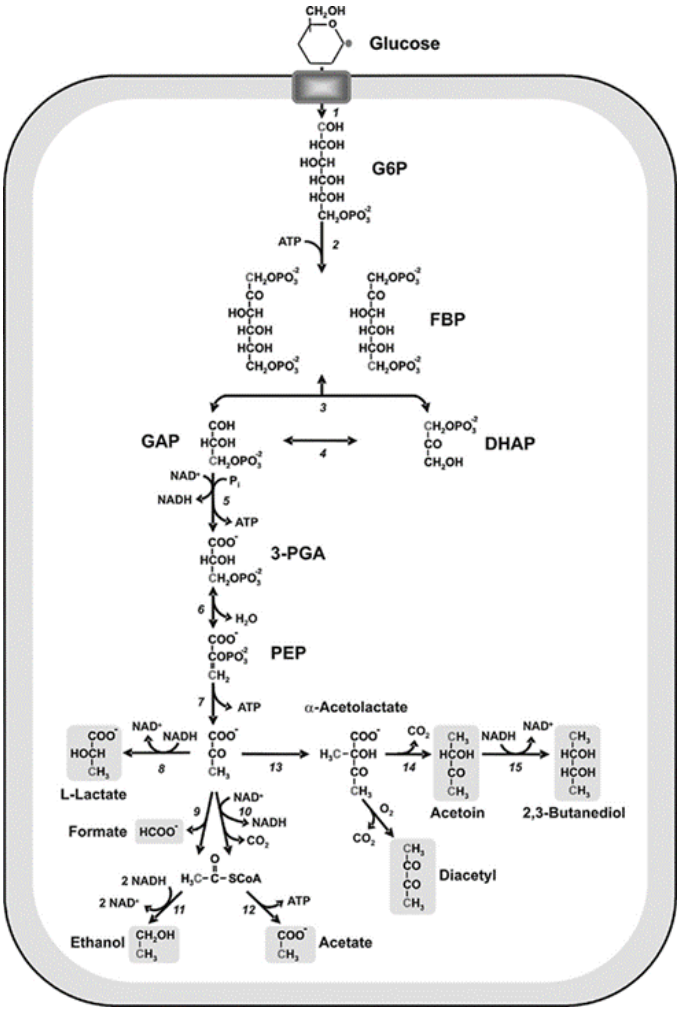


**Figure 1.2. Schematic representation of the pathway for glucose metabolism and pathways downstream of pyruvate in *L. lactis*.** Expected labeling pattern of glycolytic intermediates and end-products derived from the metabolism of [1-13C]glucose is shown. It should be stressed that, under anaerobic glycolysis, lactate accounts for about 95% of the label from glucose. The reactions indicated are catalyzed by the following enzymes: 1. phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS, *ptnABCD*) or/and non-PTS permeases (genes unknown)/glucokinase (*glk*) system; 2. phosphoglucose isomerase (*pgi*) and 6-phosphofructo-1-kinase (*pfk*); 3. fructose 1,6-bisphosphate aldolase (*fba*); 4. triosephosphate isomerase (*tpi*); 5. glyceraldehyde 3-phosphate dehydrogenase (*gapB*) and phosphoglycerate kinase (*pgk*); 6. phosphoglyceromutase (*pmg*) and enolase (*enoA*); 7. pyruvate kinase (*pyk*); 8. lactate dehydrogenase (*ldh*); 9. pyruvate-formate lyase (*pfl*); 10. pyruvate dehydrogenase complex (*pdhABCD*); 11. acetaldehyde dehydrogenase (*adhE*) and alcohol

dehydrogenase (*adhE*); 12. phosphotransacetylase (*pta*) and acetate kinase (*ackA*); 13. a-acetolactate synthase (als); 14. a-acetolactate decarboxylase (*aldB*); 15. 2,3-butanediol dehydrogenase (*butB*). Reprinted with permission from (29), Copyright (2005) Federation of European Microbiological Societies.

**Energy system**

As a key step in energy metabolism, adenosine triphosphate (ATP) is synthesized as energy currency from the energy carried compounds, mostly are sugars like glucose, fructose, and lactose, etc. Unlike bacteria that possess a functional electron transport chain (ETC), which could synthesize ATP via oxidative phosphorylation, lactococcus instead synthesize ATP via substrate level phosphorylation (reaction 5 and 7, Figure 1.2). Interestingly, even when *L. lactis* was supplied with hemin, which could construct a simple ETC in cells, it still mainly uses substrate level phosphorylation for ATP synthesis (30). Thus, from this aspect, it could be an advantage for engineering $NAD^+$ regeneration in *L. lactis* without dramatically disturbing ATP/ADP ratio in cells. Although the $H^+$-ATPase in *L. lactis* is not for ATP synthesis, it is important for maintaining the intracellular pH, therefore, is indispensable for cell growth (31). Because pumping proton by $H^+$-ATPase is an ATP driving process, when hemin is supplied, higher biomass could be observed due to the ATP saving by the ETC. By overexpressing F1-ATPase in *L. lactis*, it could decouple glycolysis and cell growth by burning intracellular ATP (32). Besides in the glycolysis process, ATP synthesis could also happen in the process of producing acetate (reaction 12, Figure 1.2). However, this pathway is mainly active and available when *L. lactis* shift into heterologous fermentation. For optimizing the fermentation process and steadily supplying ATP in a specific situation, simply adding arginine could be implemented. This is because *L. lactis* could metabolize arginine via the arginine deiminase pathway, which results in extra ATP along with ammonia, ornithine, and carbon dioxide as byproducts (33).

**General features of sugar metabolism**

*L. lactis* are semi-anaerobic species and not able to complete the tricarboxylic acid (TCA) cycle. Instead, *L. lactis* catabolize sugars to pyruvate via the glycolytic pathway and gaining ATP from substrate level phosphorylation (34). From this process, cells gain 2 net ATP and 2 net NADH. There are three ways for sugar uptake in *L.lactis*, via permease (coupled to ion translocation), via carbohydrate transport ATPase (ABC transport system), and via carbohydrate phosphotransferase system (PTS), where the latter one is the primary sugar

transportation system for most of the sugars (29). Because the PTS system transport sugars by consuming phosphoenolpyruvate (PEP), it is commonly called the PEP-PTS system. For instance, *L. lactis* uptake one glucose by PEP-PTS system with the consumption of one PEP, forming Glucose-6 phosphate (G6P). Most of the other sugars transported by PEP-PTS or permeases are also catabolized to form G6P by related enzymes to continue the subsequent glycolysis reactions. Whereafter, G6P was catabolized by other enzymes in the glycolysis pathway and subsequently forming pyruvate.

**$NAD^+$/NADH redox balance in *L. lactis***

Many biological processes need reductive or oxidative agents to accomplish their reactions. In *L. lactis*, $NAD^+$/NADH are the most used redox cofactors that responsible for electron transfer among catabolic substrates. The level and ratio of $NAD^+$/NADH are demonstrated to be relevant for many enzyme activities, such as GAPDH and LDH (35). Due to their significant role in bioactivities, the level and ratio of $NAD^+$/NADH were considered as important indicators for cell redox status. In *L. lactis*, NADH is mainly produced from the glycolysis pathway. To achieve the redox balance in cells, in the presence of oxygen, NADH could be oxidized by NADH oxidase (Nox) to $NAD^+$, however, this may result in oxidative stress in cells. In this case, reconstituting the respiration chain by adding hemin could relieve oxidative stress (36). While in the absence of oxygen, NADH is predominantly oxidized by pyruvate, generating lactate and $NAD^+$. Besides, NADH could also be oxidized by other substrates like acetyl-CoA or acetoin, forming ethanol or 2,3-butanediol respectively. Due to this, *L. lactis* could be engineered to produce many different compounds by rewiring the NADH flux in cells. From different growth condition or different genetic background, *L. lactis* could shift itself to meet the NADH and ATP balance in cells, and finally forming different products, such as acetate, ethanol, lactate, acetoin, etc.

**Homofermentative metabolism**

The *L. lactis* could grow well under anaerobic condition. In anaerobic conditions, to meet the NADH/$NAD^+$ balance, the oxidation of NADH is coupled with the reaction of converting pyruvate into compounds like lactate, ethanol, and 2,3-butanediol. When supplying *L. lactis* with rapidly metabolized sugar, like glucose, *L. lactis* would metabolize more than 90% of sugars into lactic acid. Lactate dehydrogenase (LDH), the enzyme responsible for converting pyruvate to lactate, is encoded by *ldh* gene, which is regulated by *las* operon together with *pfk* and *pyk* gene (37). Interestingly, in *L. lactis*, there are two structural-similar proteins for *ldh*,

which are *ldhX* and *ldhB*, where the last one has been proved to contribute to LDH activity in *ldh* deleted strain. Since different products could be formed from pyruvate, the fact that cells predominantly producing lactate as product suggests a high activity of LDH and comparative low activity of the competitive enzymes in anaerobic condition. It has been demonstrated that the LDH activity is controlled by the NADH/$NAD^+$ ratio in cells, where a complete inhibition of LDH was detected when NADH/$NAD^+$ ratio below 0.03 (38).

**Heterofermentative metabolism**

*L. lactis* could shift itself into heterofermentative metabolism in several conditions, such as in an oxygen-rich environment and on a slow metabolized carbon source. The shift of metabolism pathway and metabolic regulation are important for a rapid response to the turbulence of environmental conditions (39). There are several enzymes that control the glycolytic flux rate, like PK, GAPDH, and FPK. The activity of PK is positively correlated to the level of FDP in cells and GAPDH is regulated by the NADH/$NAD^+$ ratio (40)(41)(35). Differently, the activity of PFK is regulated by the ATP level in cells, as low ATP/ADP ratio leads to a reduction of PFK activity at least two-fold (42). When *L. lactis* grow in slowly metabolized sugars, such as galactose and maltose, the glycolytic flux and NADH/$NAD^+$ level would be decreased, resulting in lower LDH activity, and higher PFL and NADH oxidase (Nox) activity in anaerobic condition or higher PDH activity in aerobic condition (39)(43). To oxidize the NADH generated from glycolysis and meet the NADH/$NAD^+$ balance, cells would produce lactate, ethanol, and 2,3-butanediol, together with formate, acetate, and acetoin. Consequently, the cells shift into heterofermentative metabolism.

## 1.3 The role of phosphorylation in PTS sugar transportation and sugar repression metabolism

*L. lactis* are able to uptake a wide repertoire of sugars and sugar alcohols, such as galactose, mannose/mannitol, glucose, fructose, maltose, and cellobiose, most of which are primarily transported by PTS system and would be finally metabolized via central sugar metabolism. Like most bacteria, *L. lactis* have a hierarchical of carbohydrate utilization in presence of different sugars. Now it is clear that many of those biological processes involve phosphoryl group transfer events, which play significant roles on regulating the expression or affecting the activity of relevant enzymes.

### 1.3.1 PTS sugar transportation system

The PTS-mediated sugar transport system usually consists of one membrane-spanning protein EIIC and four cytoplasmic proteins EI, Hpr, EIIA, and EIIB, where the last one is usually fused with EIIC to form EIIBC complex. For every sugar, cells equipped with a set of corresponding PTS components. Nevertheless, Hpr and EI are general PTS components for every PTS sugar, while EIIA and EIIBC are usually not shared among different sugars. In addition, the EIIA and EIIBC for a specific sugar are usually located in the same operon, which may include a set of gene regulators and genes encoding related enzymes.

PTS uses phosphoenolpyruvate (PEP) as phosphoryl group donor for phosphorylating the carbohydrates, thus, the PTS is also called PEP-PTS. Once sugar is phosphorylated by its sugar-specific EIIBC complex, forming sugar-phosphate, it will undergo the central sugar metabolism, thus, the phosphoryl group transfer plays a central role in the PEP-PTS system. The phosphorylation begins from EI, which is encoded by *ptsI* gene. EI is a conserved enzyme across different bacteria and it could be auto-phosphorylated in the presence of $Mg^{2+}$ in its conserved Histidine (44)(45)(46). Once EI is phosphorylated, it will transfer its phosphoryl group to Hpr, which plays a key role in regulating the expression of genes encoding PTS components. In most of the low GC gram-positive bacteria, Hpr has a conserved histidyl reside, His-15, which could be phosphorylated by EI or dephosphorylate by EIIA (Figure 1.3) (47)(48)(49). In these bacteria, there is another conserved active site in Hpr, which is a seryl residue at 46 position from N terminal, so addressed Ser46. Instead of being phosphorylated by EI as His15 does, Ser46 is phosphorylated by Hpr kinase with ATP consumed, causing lower affinity with EI and carbon catabolic repression (CCR) for many PTS sugars (50). Although the phosphorylation in Ser46-Hpr would affect the phosphoryl group transfer among PTS components, it is not a part belongs to this process, and it would be discussed in the next section. Sugar specific EIIAs are the phosphoryl group acceptor for the P~His15-Hpr, and it would deliver the phosphoryl group to sugar specific EIIBC complex and finally to the their corresponding sugars. The number of the genes encoding EIIABC determines how many kinds of sugars the cells could uptake through PTS transportation system. PPE-PTS system uptakes sugars by consuming one PEP, which means cell would loss one potential ATP catalyzed from pyruvate kinase. However, compared with other sugar transportation system, this is the lowest cost for introducing sugars into glycolytic pathway (51).

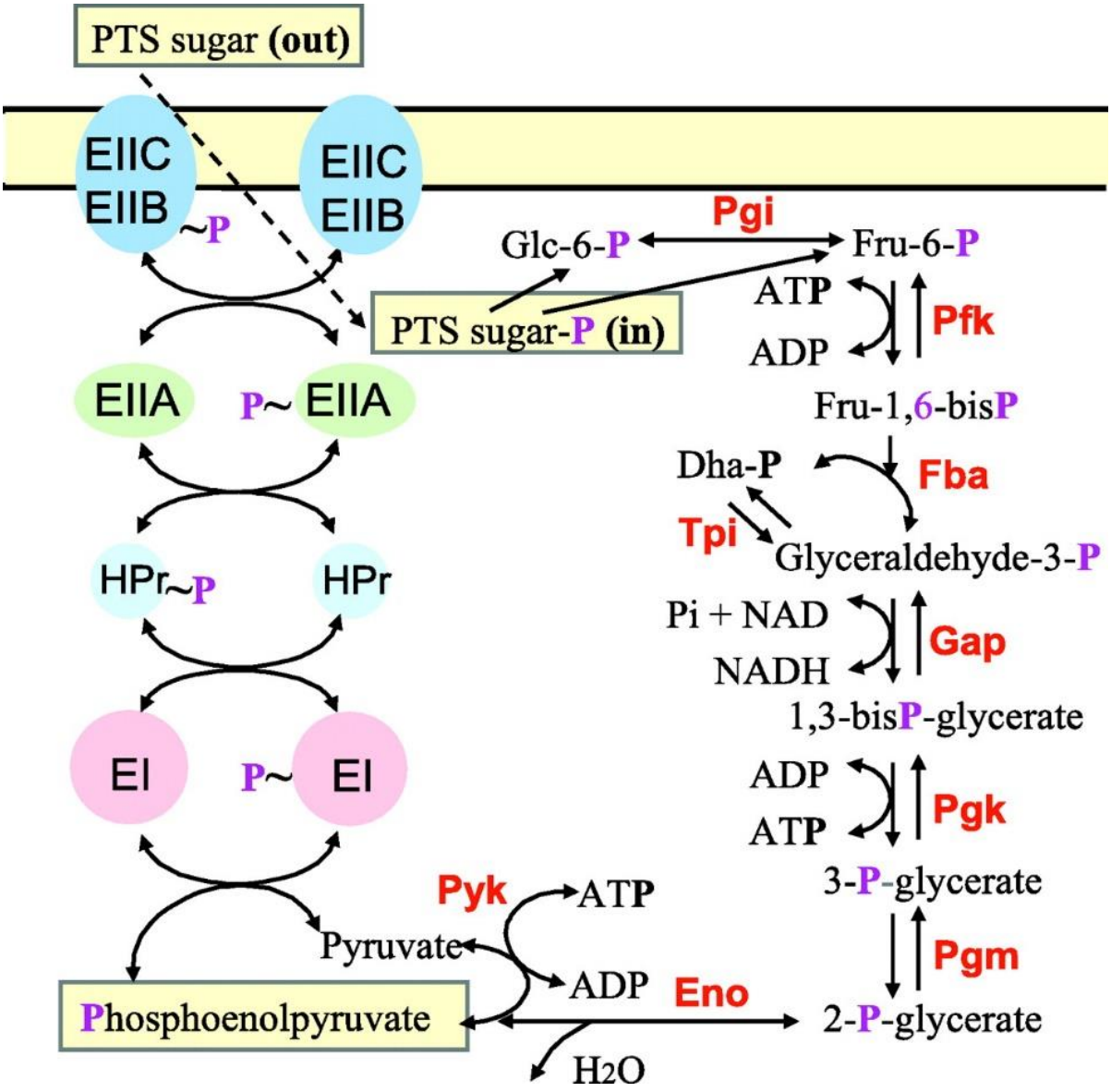


**Figure 1.3. Carbohydrate transport and phosphorylation by the PTS and their coupling to glycolysis.** Carbohydrates are transported and concomitantly phosphorylated by the PTS. The phosphorylated carbohydrate feeds into glycolysis, normally at the glucose-6-P or fructose-6-P level. Two phosphoenolpyruvate molecules are usually formed in glycolysis, one of which is used to drive the transport and initial phosphorylation of the carbohydrate. As a result, the phosphorylation state of the PTS proteins depends on both the concentration of extracellular carbohydrates and the ratio of internal phosphoenolpyruvate and pyruvate. Abbreviations for enzymes (in boldface type) are as follows: *Pgi*, phosphoglucose isomerase; *Pfk*, phosphofructokinase; *Fba*, fructose-1,6-bisphosphate aldolase; *Tpi*, triose-phosphate isomerase; *Gap*, glyceraldehyde-3-phosphate dehydrogenase; *Pgk*, phosphoglycerate kinase; *Pgm*, phosphoglycerate mutase; *Eno*, enolase; *Pyk*, pyruvate kinase. Reprinted with permission from (44), Copyright (2006) American Society for Microbiology.

### 1.3.2 Carbon catabolic repression

Carbon catabolic repression (CCR) is a common regulatory mechanism in bacteria that allows them prior to use the easily metabolized carbohydrates than less favorable ones (52). In low GC content Gram-positive bacteria, like *L. lactis* and *B. subtilis*, P~Hpr-Ser46 plays an important role in CCR. Although P~Hpr-Ser46 does not serve sugar uptake and the phosphorylation chain, it has several regulatory functions for affecting the relevant enzymes activity (Figure 1.4). The phosphorylation status of Hpr-Ser46 is affected by the bifunctional enzyme activity of Hpr kinase/ phosphorylase (HprK/P), which would respond to the alterations of ATP, Pi, PPi, and FBP levels in cells (44). P~Hpr-Ser46 primarily exerts its function as a corepressor for CCR and as an activator for catabolite control protein A (CcpA) (53)(54). When Hpr is phosphorylated in its Ser46, although there is no big change in the protein structure, the affinity between Hpr and EI would be decreased, further resulting in a decreased level of P~Hpr-His15 in cells (55). Moreover, P~Hpr-Ser46 could bind to CcpA forming P~Hpr-Ser46/CcpA complex. This interaction is highly specific since unphosphorylated Hpr or P~Hpr-His15 presented poor affinity to CcpA (54)(53). P~Hpr-Ser46/CcpA then would bind to the catabolite response elements (cre), which are DNA sequences with high similarity, usually locates just in the upstream of the start codon and overlapped with the promoter elements (56), causing CCR. For example, when fast metabolized sugars like glucose present, the P~Hpr-His15 level in cells would decrease due to the high phosphoryl group demand from its acceptor EIIA, instead, the high FBP pool in cells would activate HprK/P to form P~Hpr-Ser46. Then P~Hpr-Ser46/CcpA complex would repress the expression of operons responsible for uptaking slow metabolized sugars, like mannitol, exerting CCR (57).

Moreover, P~Hpr-Ser46 regulates sugars uptaking not only by forming P~Hpr-Ser46/CcpA complex but also by dependently interacting with the related machinery, called inducer exclusion (58), which described the inactivation of sugar-transport related permeases when rapid metabolized sugar, like glucose present. In *L. brevis*, P~Hpr-Ser46 has been approved to bind to the galactose/$H^+$ symporter causing inducer exclusion (59). A similar phenomenon was also detected for maltose and ribose in *L. lactis* (57).

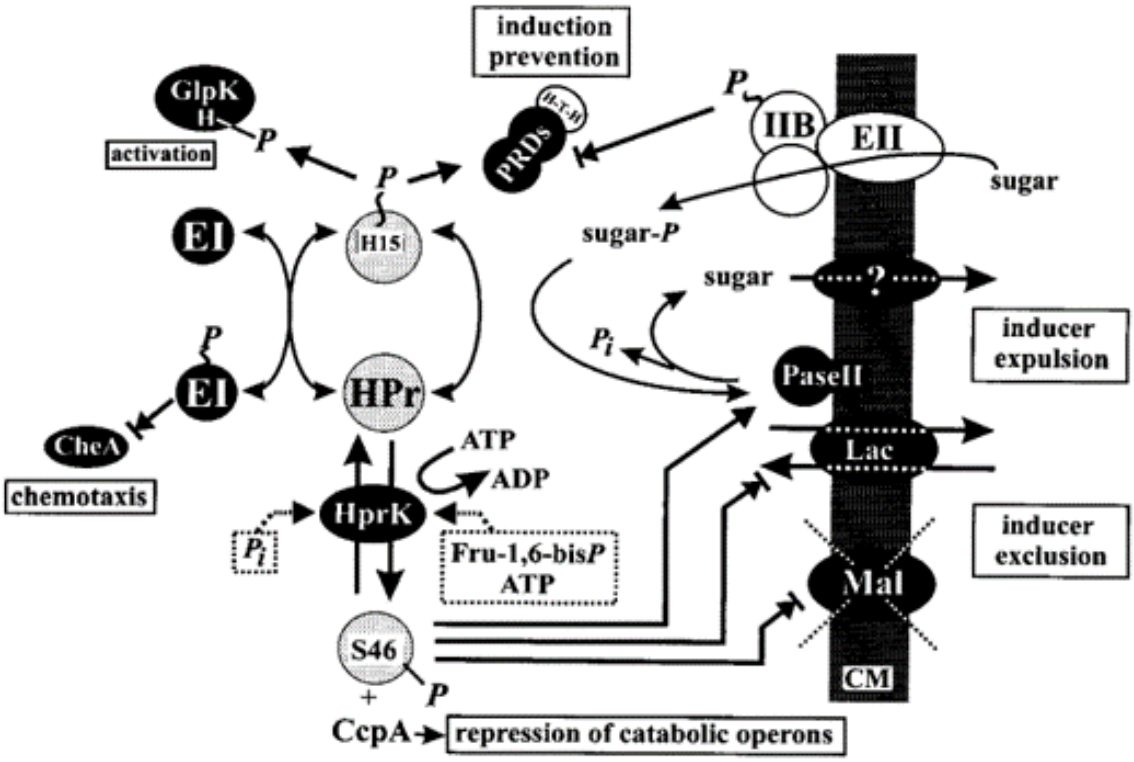


**Figure 1.4**. **CCR and inducer control in low-GC Gram-positive bacteria.** On a high free energy potential of the cell becomes the HPr protein phosphorylated at Ser-46 by ATP-dependent HPr (Ser) kinase/phosphatase (HprK). The reverse reaction occurs when the energy potential of the cell de- clines (sensed by Pi levels). The HPr (S46-P) acts as a corepressor to the global catabolite control protein, CcpA. The Hpr (S46-P) may also allosterically inhibit specific non-PTS permeases (e.g., maltose: $H^+$ symporter of *L. casei*; Mal), thereby preventing sugar accumulation (inducer exclusion). Conversion of an active transporter to diffusion facilitator (e.g., lactose: $H^+$ symporter of *L. brevis*, Lac) will lead to inhibition of transport (inducer exclusion) and concomitant release of pre-accumulated sugar (inducer expulsion). The HPr (S46-P) may stimulate activity of specific carbohydrate-P phosphatase (PaseII), thereby triggering the transport of excessive amounts of carbohydrate from the cell by uncharacterized transporter (inducer expulsion). In the absence of inducer (glucose) the HPr (H15-P) can stimulate glycerol kinase (GlpK) and particular transcriptional activators and antiterminators (Table 2) by specific phosphorylations. The operon-specific positive transcriptional regulators are inhibited by phosphorylation by cognate IIB-P abundant on the absence of the transported carbohydrate (induction prevention; PRD: PTS regulation domain, H-T-H: helix-turn-helix DNA binding motif of transcriptional activator). In B. subtilis, the chemotaxis towards the PTS carbohydrates is mediated primarily by the methyl-accepting chemotaxis protein, McpC. The response to negative signal, i.e., moving of the cell down the carbohydrate gradient, seems to be amplified by transient increases in EI-P levels which inhibits chemotatic sensor kinase, CheA. Reprinted with permission from (60), Copyright (2001) Elsevier.

### 1.4 Compounds and food ingredients produced by *L. lactis*

*L. lactis* could produce a wide range of compounds, which contribute to flavor and texture factors. Besides the applications in the dairy and food industry, *L. lactis* is also regarded as an excellent cell factory for producing valuable compounds like lactate, 2,3-butanediol, diacetyl, folate, etc. This makes *L. lactis* a highly industrially important bacterium and shares a multi-billion dollar market worldwide (61).

#### 1.4.1 Production of biofuels

**Ethanol**

Ethanol is a great alternative for fossil fuel and dominates the market in biofuels. *L. lactis* equipped with all necessary genes for producing ethanol but could only produce a small amount of ethanol during heterologous fermentation, probably because of the low activity of the related enzymes and the competitive metabolic pathways. For improving the ethanol production in *L. lactis*, Solem et al. developed a strain with deleted *ldh*, *ldhB*, *ldhX*, *pta*, and *adhE*, and with complementary PDC and AdhB from *Z. mobilis*. This strain could ferment 87% metabolized sugars into ethanol (62). After introducing a lactose metabolism, it could produce 41 g/L ethanol from cheap substrate corn steep liquor (63), which demonstrated the great potential for producing ethanol from *L. lactis*.

**Butanol**

Butanol has many important applications, for example, as a solvent and an intermediate in chemical synthesis. Besides, it is also regarded as a biofuel alternative due to its high energy density and lower hygroscopicity compared with ethanol (64)(65). High butanol isomers tolerance for *L. lactis* was reported with a concentration of around 20 g/L, which could be further improved to higher than 40 g/L by laboratory evolution (66)(67). Although *L. lactis* do not equip with all necessary enzymes for producing butanol, several attempts have been reported for developing a mutagenic strain. Liu et al. achieved 28 mg/L butanol in *L. lactis* by introducing a thiolase from *Clostridium beijerinckii* P260 (68). Recently, 5.9 g/L 2-butanol production with 58% yield was achieved by using a 4:1 *L. lactis-L brevis* co-cultivation system, which is the highest titer and yield for butanol production from a single glucose fermentation setup ever reported.

### 2,3-Butanediol

2,3-Butanediol is regarded as a promising biofuel, raw material for making plastics, perfumes, and pharmaceuticals (69). *L. lactis* could form 2,3-butanediol in two ways, one is catalyzed from acetoin another is from diacetyl. By overexpressing acetoin reductase (ButA) and α-acetolactate synthase (Als), Gaspar et al. achieved 67% yield of 2,3-butanediol from an LDH-deficient strain, with formate and ethanol as by-products (70). Liu et al. introduced *Enterobacter cloacae* butanediol dehydrogenase (EcBDH) in a multiple engineered *L. lactis* strain (deficient in LDH, PTA, ADHE, ButBA) and finally achieved 51 g/L with 47% yield of 2,3-butanediol from whey permeate (71). The efficient production of 2,3-butanediol demonstrated the potential of using *L. lactis* as a robust cell factory for producing biochemical.

## 1.4.2 Production of food ingredients

### Lactic acid

Lactic acid is a primary product from *L. lactis*, and is widely used in food and pharmaceutical industries. Lactic acid contributes to special flavor and texture in dairy products. Additionally, Lactic acid contributes to an acid environment that would further inhibit the growth of other microbes and further prolong the shelf-life of the products. *L. lactis* exhibits strong tolerance to lactic acid and is able to produce around 100g/L lactate with high productivity of 3.0g/L/h (72). Furthermore, *L. lactis* could produce lactate from a wide range of substrates, including cheaper ones like lignocellulose hydrolysates and xylose, which makes *L. lactis* is a great producer for Lactate (73). Lactate has two isoforms, D-lactate and L-lactate, where the latter one is the main form produced from *L. lactis*, however, a recent study showed engineered *L. lactis* could produce 15.0 g/L D-lactate after 24h cultivation by using starch as substrate (74).

### Diacetyl

Diacetyl acts as an aromatic compound and gives the buttery flavor for dairy products, such as butter and cheese. In *L. lactis*, diacetyl could be generated from pyruvate by two steps, where the first step is catalyzed by α-acetolactate synthase to generate α-acetolactate. Then α-acetolactate would be spontaneously decomposed to form diacetyl since α-acetolactate is chemically unstable (75). There are several attempts for enhancing diacetyl production in *L. lactis*. By selection of mutants with inactive α-acetolactate decarboxylase and low LDH activity, it was shown 6 mM diacetyl could be generated when growing in milk with catalase supplemented (76). Instead, enhance NOX activity would also help cells to bypass the lactate

generation, because the pyruvate pool would be rerouted to NADH-independent pathways in this case. By implementing this strategy, around 80% diacetyl was achieved under aerobic conditions by using an α-acetolactate decarboxylase deficient strain (77). More recently, Jianming and co-workers exploited a new strategy by combining metal-ion catalysis, metabolic engineering, and respiration controlling techniques, which achieved 95 mM diacetyl production with 87% yield (78).

**Alanine**

Amino acids production shares a big market in the world. As one of the major amino acids, alanine is predominantly used in most food and pharmaceutical industries. As a food-grade strain, *L. lactis* is an attractive cell factory for producing alanine. One study showed *L. lactis* is possible to efficiently produce alanine by combining rerouting *Bacillus sphaericus* alanine dehydrogenase (AlaDH) and rerouting the $NAD^+$ flux (79). By further optimizing pH and ammonium concentration, this strain could produce around 200 mM alanine as the only product. Interestingly, by solely complementing *Bacillus subtilis* AlaDH, it was reported by Ye et al. that NZ9000 could achieve around 580 mM alanine, which is 26 times more than that from its parental strain (80). This was claimed because *Bacillus subtilis* AlaDH is much competitive for the substrate pyruvate than native LDH in *L. lactis*.

**Acetoin**

Acetoin serves as an aroma in dairy products and therefore is an industrially important compound. *L. lactis* could form acetoin through its native pathway by catalyzing pyruvate with ALS and ALD together. By inactivating the competing product pathways, Liu et al. finally obtained a homo-acetoin producing strain with around 78% yield from glucose (81). In that work, to improve acetoin production, cell growth was decoupled by overexpressing $F_1$-ATPase and finally could achieve a titer of 14 g/L acetoin from cheap dairy waste. Furthermore, Liu et al. reported that the isomer of acetoin, (3S)-acetoin, could be produced from *L. lactis* by introducing diacetyl reductase and metal ion catalyst together, giving 66 mM with 71% of the theoretical maximum yield (82).

**Acetaldehyde**

Acetaldehyde is responsible for yogurt flavor and aroma therefore is a highly important compound for dairy products. In *L. lactis*, Acetaldehyde could be naturally converted from acetyl-CoA catalyzing by aldehyde dehydrogenase (Adh). However, acetaldehyde could be

hardly produced as an end product because it could be subsequently converted into ethanol. For enhancing acetaldehyde production in *L. lactis*, Bongers et al. introduced *Zymomonas mobilis* pyruvate decarboxylase, which could convert pyruvate directly into acetaldehyde, and meanwhile, overexpressed NADH oxidase (Nox) to maintain a lower $NADH/NAD^+$ ratio in cells, and finally rerouted almost 50% of the consumed glucose into acetaldehyde (83).

**Riboflavin**

Riboflavin also called vitamin $B_2$, plays a key role in cellular metabolism since it works as the precursor of Flavin adenine dinucleotide and coenzymes Flavin mononucleotide, which are essential biomolecules for cellular redox reactions involving enzymes like NADH (84). In *L. lactis* genome, rib operon (*ribGBAH*) is responsible for generating riboflavin. By overexpressing rib operon, *L. lactis* could efficiently produce riboflavin with a titer of 24 mg/L (85). Instead, by evolving *L. lactis* with roseoflavin, which is an analog and competitor for riboflavin, Burgess et al. obtained several spontaneous riboflavin overproducing mutants, which could achieve a significant high riboflavin production, around 900 mg/L (85). For applying riboflavin production in dairy products, Chen et al. obtained a riboflavin overexpressing strain by combining roseoflavin adaptation, random mutagenesis, and microfluidic screening, which could increase riboflavin content in milk to 2.81 mg/L (86). These results demonstrated that *L. lactis* could be a great host for producing riboflavin.

**Folate**

Folate also called vitamin B9, is an essential dietary nutrient. Folate is responsible for delivering one-carbon units in lots of biological reactions, such as biosynthesis of pyrimidines, purines, and amino acids. In *L. lactis*, a folate gene cluster containing genes *folA*, *folB*, *folKE*, *folP* and *folC* was identified by Wilbert and co-workers (87). Their study further showed that overexpressing *folKE* resulted in 10-fold higher of extracellular folate and 3-fold higher of the total produced folate. Additionally, overexpressing *folA* resulted in at least 2-fold lower folate production, suggesting a negative feedback effect for folate biosynthesis by *folA*. Wegkamp et al. claimed that folate production in *L.lactis* could be enhanced by overexpressing *pABA* and folate gene clusters together, achieving 2.7 mg/L, which is around 80-fold higher than that from wild type strain (88).

## 1.5 Tools for fundamental and applied researches in *L.lactis*

### 1.5.1 Adaptive laboratory evolution (ALE)

The original concept and principle for ALE could be traced to the mid-19$^{th}$ century, when the importance of natural and artificial selection for biological evolution & breeding and germ theory were addressed by noble scientists like Charles Darwin, Louis Pasteur and Robert Koch, etc. (89). However, ALE was limited to theoretical studies because of lacking experimental approaches for a long time (90). The achievements of sequencing and omics analysis technologies during the last two decades allow the fast development of concepts and tools for studying biological genomic and metabolic features. ALE became one of these powerful tools based on studying the correlation between the phenotypical changes and the geomics changes, which could be obtained by specifically controlled laboratory evolution strategies (91).

**Principles and establishment for microbial ALE**

Studying microbial cells by using ALE offers several advantages: simple nutrients requirements, general regards as non-GMO approach, easy to implement and fast growth and short interval between generations. The fast growth particular for bacteria cells makes researchers easily obtain several hundred generations within few weeks. ALE is based on the occurrence of the single nucleotide variation, deletion, insertion genomic events, or other genomic events during propagation. Some of these genomic modifications lead to new phenotypes, which would help the cell fit the selective environment. During the ALE, the phenotypes that fit the selective environment would accumulate rapidly and would finally dominate the whole population. A typical microbial ALE experiment spans between 100 to 2000 generations, which would take few weeks to few months (89). There are several ways to establish an ALE (Figure 1.5), the traditional one is using batch culture by regularly diluting the cells into a fresh culture medium. This approach is easy to operate and cheap since it could be established by the most commonly used vessels in the lab. The second way is to use chemostat system, which enables constant cell density and growth phase during the ALE. Another way for ALE is to use an emulsion culture and selection system. This approach bases on the establishment of small cells containing vesicles by mixing the culture medium and oil together, leading to a huge number of replicates and selections.

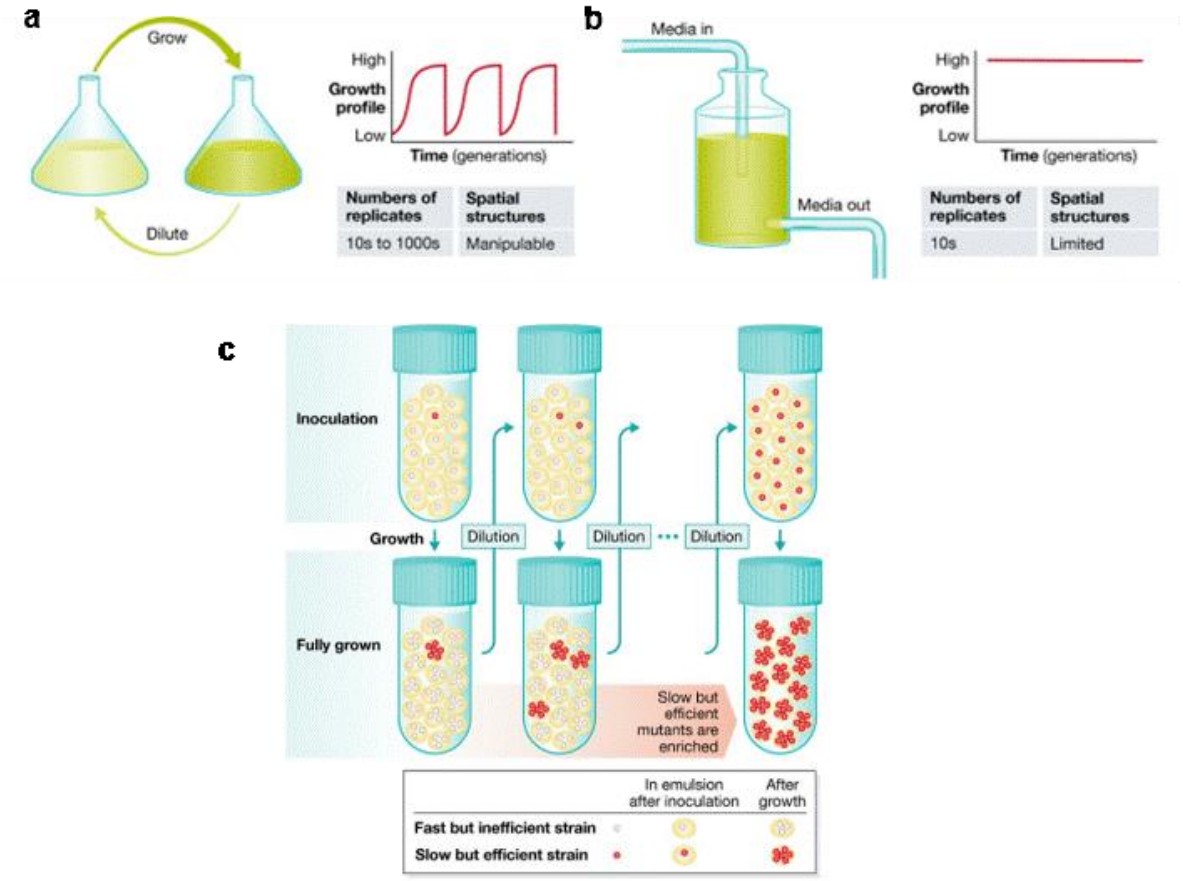


**Figure 1.5. Different establishments for ALE.** a, Different establishments of ALE. b, Chemostat culture. c, Emulsion culture system. Reprinted form (92). Copyright (2019) John Wiley and Sons.

## Challenges for ALE

Although ALE has been a powerful strategy for studying microbes, it has some limitations. One is the limited time scale. Some of ALE could be very fast, however, sometimes the evolutionary process may extremely slow to see a desired phenotype, longer than a researcher's grant span, and even professional lifetime (93). Because ALE relies on the occurance of random genomic events, it makes the dominant phenotype sometimes not the one people desired or the one with the highest fitness under the selective pressure, but the phenotypes that make cells able to live with the least trade-offs. Thus, a good selection strategy with proper selective pressure is very important for ALE. In addition, another challenge is ALE relies on the basic genome constitute. This means it is extremely difficult for microbes to evolve a phenotype if there is no such a gene or genomic basis associated with that phenotype, even with a perfect experimental design. In this case, the ALE process could be very long and risky. However, it is hard to say if an ALE would be a success or not or how long it will last before

we do, as microbes are smart organisms and the knowledge on their biological mechanism is limited. Sometimes finding a good ALE experimental strategy and proper selective stress could be also challenging. Selective stress could be principally divided into two categories, i.e., nutrients stress and environmental stress. The selective pressure for ALE should be in a proper range, high selective pressure might make cells hard to live or lead to undesired evolutionary directions. On the contrary, low selective pressure might lead to low evolution efficiency and a long ALE process, because cells need to change nothing. All in all, a preliminary understanding of the aimed subjects and a well-designed ALE strategy are very important.

**Achievements in *L. lactis***

Several studies have been reported for applying ALE in *L. lactis*, for example, adapting to heat stress, which is an important feature in industrial processes. *L. lactis* suffering high temperatures during the syneresis step in making semi-hard cheeses, resulting in changes in the physiological properties. Chen et al. obtained a non-GMO thermal-tolerant variant by adapting MG1363 in 38-40 degrees after 860 serial transfers (94). This variant has 33% faster growth and a 12% higher specific lactate production rate than its parental stain at 38 degrees, which is the highest permissive temperature for MG1363. Genome sequencing showed several mutations happened in the genes that may relate to the thermal tolerance, such as *ribU* and *rpoC*, which are critical for riboflavin and fatty acid synthesis in *L. lactis*. Cell robustness also important for cheese production. For having a better performance during the industrial process, Maria et al. obtained seven *L. lactis* variants after adapting cells under cells envelope stress by adding bacteriocin Lcn972 (95). Although some of the variants showed plasmid loss and higher sensitivity to phages after adaptation, they got one variant, Lcn972R, which showed less autolytic and much robust to cell wall-active antimicrobials. In another case, as *L.lactis* is regarded as a potential platform for producing isobutanol, which is a promising biofuel. To enhance the isobutanol tolerance for *L. lactis*, Jaya et al. obtained a variant that could tolerate up to 40 g/L of isobutanol after 60 days ALE process (67). Genomics analysis revealed 13 convinced genome modifications in the evolved strain, which are deduced to critical for membrane transport, cell wall synthesis, sugar uptake, and amino acid metabolism.

### 1.5.2 Artificial genetic modification techniques

#### Homologous recombination

Recombinant DNA construction is a strong and traditional tool for modifying bacteria genome (deletion, insertion, substitution). Bioengineering *L. lactis* with homologous recombination relies on the RecA-dependent homologous paring and exchanging events and firstly accomplished in 1989 (96)(97). RecA is a recombinase that relates to the mechanism of DNA double-strand break (DSB) repair, which presents in lots of bacteria and pretty conserve cross species (98). RecA could polymerize on DNA, which would promote the process of forming a helical filament for homologous pairing and of the exchange reaction for generating a hetero-duplex DNA (99)(100). This mechanism has further developed into a powerful genome-editing tool for prokaryotic organisms, including *L. lactis*, by using non-replicative or conditional replicative plasmids with a double-crossover process (described in Figure 1.6). A routine protocol for modifying *L. lactis* genome by using homologous recombination is around 3 weeks due to the rare occurrence of recombination events and laborious works in the sub-cultivation and screening steps (101). Nevertheless, it is still the primary genome-editing tool for *L. lactis* because of its stable performance and markless features.

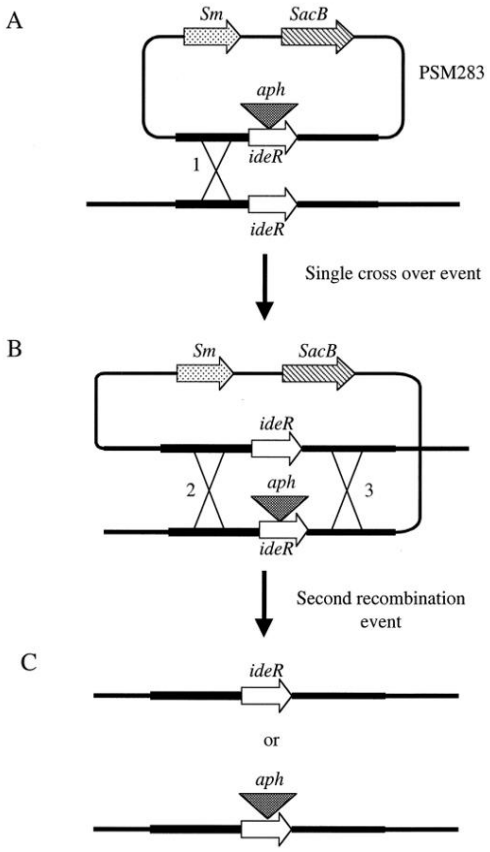


**Figure 1.6. Scheme of double-crossover process.** Example from Construction of single-crossover and double-crossover strains of *M. tuberculosis* using plasmid pSM283. (A) The nonreplicative plasmid pSM283 was transformed into M. tuberculosis. Selection with kanamycin and streptomycin allows growth of strains in which pSM283 was inserted in the chromosome by a single crossover. The single crossover can occur at homologous regions on either side of the kanamycin cassette. Crossover at position 1 is illustrated. (B) A second recombination event between duplicated sequences leads to resolution of the duplication and loss of the plasmid sequences. (C) Depending on where the second recombination event occurs, i.e., interval 2 or interval 3, it can result in either restoration of the wild-type gene (interval 2) or a mutant strain (interval 3) in which allelic exchange has occurred. Reprinted with the permission from (102), Copyright (2002) American Society for Microbiology.

## CRISPR-Cas technique

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system is a powerful tool for removal or replacement of genetic elements and has been broadly exploited into various prokaryotes and eukaryotes, including LAB. CRISPR-Cas system is prevalent in *Streptococcus*, *Lactobacillus* and *Bifidobacterium*, with an occurrence rate exceeding 45% (103)(104)(105). However, CRISPR-Cas systems are significantly different among species. Some LAB species

possess very few CRISPR-Cas systems with a low occurrence rate, some even lacking complete CRISPR-Cas system, like *L. acidophilus* and *L. paracollinoides* (103). Although several achievements could be found for other LAB species (106)(107), the application for CRISPR-Cas systems in *L. lactis* is still in the early stage of adoption. To date, there is no endogenous CRISPR-Cas system has been found in *L. lactis*. Nevertheless, it was shown *L. lactis* could still be engineered by introducing *Streptococcus pyogenes* Cas9, and could be used for mutagenesis screening (108)(109). Recently, a new approach for editing *L. lactis* genome with CRISPR-Cas9 was developed introducing *Enterococcus faecalis RecT* and SpyCas9 targeting machinery into *L. lactis*. With this modulation, efficient counterselection, mutants with point mutations, and short chromosomal deletion or insertion with more than 75% recombinant efficiency during 72 hours of experimental practice could be achieved (101).

**Inducible gene expression system**

Inducible gene expression systems are powerful tools for manually regulating the expression of an interested gene and avoiding cytoplasmic accumulation of the gene products as constitutive promoters do. Several inducible expression systems has been developed in *L. lactis* and has later applied in other LAB for controlling the expression of genes (Figure 1.7).

**Nisin-controlled gene expression (NICE) system**

NICE system normally consists of a membrane-located histidine kinase NisK, an intracellular response regulator protein NisR, and *NisA* promoter (110). NZ9000, a laboratory strain derived from MG1363, integrated nisRK genes in its genome, could expression genes constructed after $P_{nisA}$, by adding a minimal amount of nisin (0.1 to 10 ng/ml). Such a simple, efficient and sensitive mechanism makes NICE system is the most commonly used inducible expression system in *L. lactis*.

**Zinc-inducible expression system**

Zinc-inducible expression system relies on the regulation mechanism of *L. lactis* zit operon, which is responsible for zinc uptake. The developed $P_{Zn}$zitR is inducible when cells suffering divalent cation starvation by adding EDTA or using chemical defined medium and strongly inhibited when excess $Zn^{2+}$ present, however, the expression level is around 5 fold lower than NICE system(111). More recently, Mu et al. developed a new zinc-inducible expression system, called Zirex, which could be induced by adding a non-toxic concentration of zinc, relying on the streptococcal promoter PczcD (112).

### Xylose-inducible expression system (XIES)

XIES is a sugar-dependent expression system developed by Miyoshi et al. in 2004 (113). This system relies on the xylose-inducible promoter, P*xylT*, and could be easily switched on by adding xylose or glucose. Comparing with NICE system, the expression level of XIES is around 10-fold lower in the exponential phase but similar to that in the stationary phase.

### Stress-induced controlled expression (SICE) system

SICE is an interesting expression system that could be induced when cells encountering harsh environments like heat shock, low pH, or high salt. It was developed by Benbouziane et al. by applying the regulation mechanism of *L. lactis* groESL operon (114). Because SICE does not require the induction culture and complementary regulatory genes, it is a promising expression system for delivering mucosal therapeutic molecules by *L. lactis*.

### P170 system

P170 system is a pH-inducible based on the expression of *L. lactis* promoter P170, which could be induced when lactate accumulated and pH reaches 6.0 or lower (113). As P170 is an auto-regulated expression system, the feature of easy to operate makes it to be a promising promoter controller applied for food-grade compounds and molecule production. As lactate will be a limitation for cell growth and production capacity during fermentation, Jørgensen et al. combined the P170 system and REED$^{TM}$ technology by removing the lactate from the culture medium and achieved high production of model protein Staphylococcus aureus nuclease with 2.5 g/L (111).

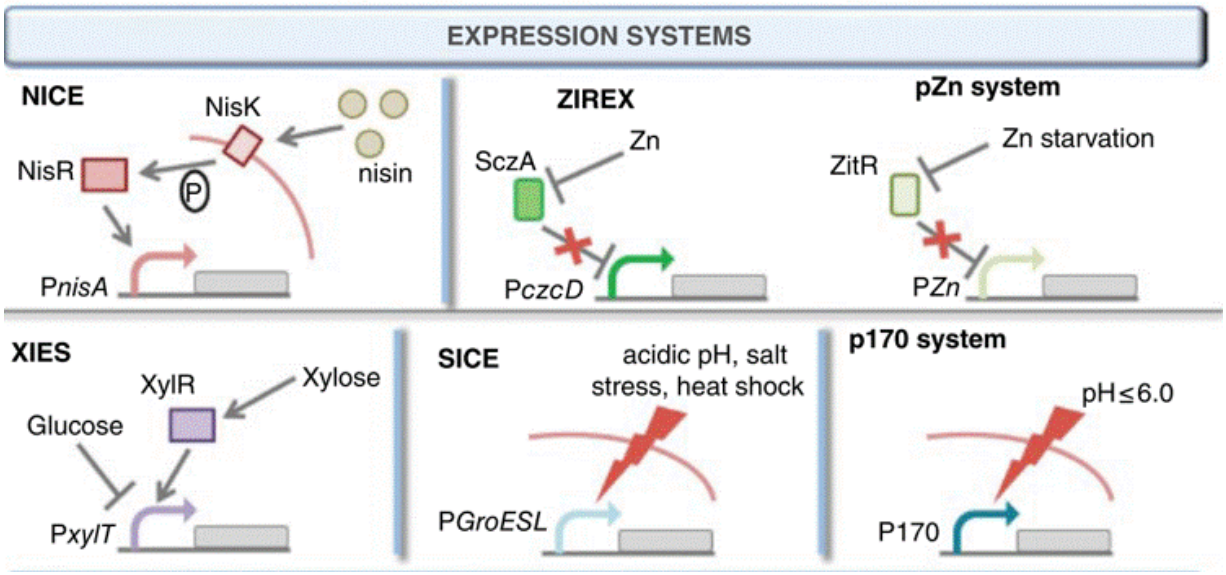


**Figure 1.7. Schematic representation expression systems applied in lactococcus.** Reprinted with permission from (16), Copyright (2016) John Wiley and Sons.

### Synthetic promoters

Synthetic promoter is an alternative for inducible promoters that could achieve desired expression level of aiming genes. To build a synthetic promoter library, a DNA sequence containing a set of minimal promoter elements is used as consensus, following by filled with random building blocks (115). A typical synthetic promoter in prokaryotic consists of a set of strong/medium -35 and -10 transcriptional elements and several degenerated nucleotides. Jensen and Hammer developed a synthetic promoter library for *L. lactis* based on a set of strong promoter elements, that described as NNNNNAGTTTATTCTTGACANNN-NNNNNNNNNNNTGRTATAATANNWNAGTACTGTT, where TATAAT is the -10 element and TTGACA is the -35 element (116)(117)(118). This library of synthetic promoters gave an alteration of promoter strength ranging from 0.3 to more than 2000 relative units, where the spacers between the consensus sequences were shown to play a key role in regulating the promoter strength (119). Although this approach could be used for modulating the expression of many genes, it appeared to be context-dependent, i.e., the strength ranking of the characterized promoters usually changes when they are constructed into a new context (120). Due to this, Christian and Jensen subsequently worked out an optimized synthetic library that was described as $RN_5AGTTTATTCTTGACAN_{14}TGRTATAATN_5$. This approach is proved to be less laborious and allows for accurate, individual, and simultaneous modulation of genes in *L. lactis* (62)(120).

### 1.5.3 High-throughput technologies

The next-generation sequencing techniques dramatically boost the development of comparative genomic studies in *L. lactis*. The first available *L. lactis* genome was provided by IL1403 in 2001 (6), while the genome information for MG1363 and NZ9000 had soon came out in 2007 and 2010 respectively (121)(122). In MG1363, around 81 pseudogenes and 2436 protein-coding genes were characterized in its 2.54 Mb genome. With the fast development of next-generation sequencing and the appearance of third-generation sequencing techniques, it is now possible to obtain both large-scale and good quality sequencing data at a low price. To date, 209 *L. lactis* genome sequences are available from NCBI assembly database. Besides, as a powerful tool for observing life activities from the transcriptional level, RNA sequencing has become an important approach for studying gene expression and regulation in *L. lactis*. Combining the analysis of genome data, RNA sequencing allows people to easily investigate gene networks and regulatory mechanisms from high-throughput data. For example, Zomer et al. predicted 82 direct targets of CcpA binding motifs based on the cre (catabolite responsive elements) sites analysis in *L. lactis* genome (56). Since CcpA is a global regulator that could play as both activator and repressor for many genes, by following investigated the transcriptional information of these genes in a *ccpA* deficient strain, they revealed that the role of CcpA for a certain gene is affected by the location of its cre site preceding the gene. As the greatest achievements of molecular biology in this century, high-through put technologies, genomics, transcriptomics, proteomics and metabolomics analysis have been become more and more usual, straightforward and convenient approaches for studying *L. lactis* respond to different growing and nutritional conditions, various stress and different genetic backgrounds.

## 1.6 Microbial production of mannitol

### 1.6.1 Mannitol and its applications

Mannitol ($C_6H_{14}O_6$) is water-soluble (213 g/L) with a molecular weight of 182.17 g/mol, and could be found in many plants, such as seaweeds and fresh mushrooms. It belongs to sugar alcohol, which is a group of polyols and is increasingly demanded in the food and medical area due to their low glycemic index, low insulin response, low calorie and sweetening traits (123). Mannitol is primarily used as a food additive and is commercially used in chocolate, chewing gums, giving sweetness and cooling tastes, thus, mannitol is also used as a pharmaceutical formulating agent (e.g., low reactivity dug and dental hygiene products) (124). Besides, mannitol also works as a versatile clinical agent. It was reported that mannitol would cause

vasodilation and increasing urinary flow by stimulating the release of vasodilatory prostaglandins in kidney (125)(126). Mannitol is a common osmotic diuretic and could be used in intravenous infusion solution with a concentration of 10% to 20%. Because mannitol could decrease renal injury and preserve the estimated glomerular filtration rate, it is commonly used for kidney transplantation and partial nephrectomies (127). Additionally, mannitol also has effects on increasing tubular flow rate, intravascular volume, and urinary output; therefore, mannitol is commonly used in vascular, hepatic, cardiac, and renal surgery. Furthermore, it was also reported that mannitol could work as an antioxidant and would scavenge hydroxyl radical, which would protect neurological functions (128)(129). More recently, it was reported that mannitol could also be used as a treatment to enhance mucociliary clearance in patients with cystic fibrosis (130). Mannitol is considered safe and is permitted by U.S. Food and Drug Administration (FDA). Toxicity studies showed mannitol has no adverse effects other than diarrhea (131). Commercially production of mannitol is primarily catalytic hydrogenation of fructose/glucose mixture. However, it is still a big problem for separating mannitol from the byproduct sorbitol in that reaction, which leads to low yield. Thus, an efficient method for producing mannitol, especially in food and pharmaceutical grade is needed.

### 1.6.2 Mannitol production from heterologous LAB and other microbes

Mannitol is mainly produced from the chemical catalyzing of fructose/glucose mixture, yielding mannitol and sorbitol, which are difficult to separate, resulting in lower yield and higher cost (132). Microbial production of mannitol would be a promising alternative since mannitol could be produced from a variety of LAB, yeast, and fungi. Mannitol production has been extensively studied by using heterologous LAB, such as *Lactobacillus* and *Leuconostoc* and *Oenococcus,* etc. Saha and Nakamura screened 72 bacteria cultures from ARS Culture Collection, where they obtained nine cultures that could produce mannitol from fructose but none of them could produce mannitol from glucose (133). They finally characterized *Lactobacillus intermedius* B-3693 as the best mannitol producer that could achieve 300 g/L mannitol within 92 h by using fed-batch fermentation from fructose. And further validated that one-third of fructose is responsible for supplying energy and could be replaced by other sugar, such as maltose, galactose, mannose, etc. Two-third of fructose is responsible for reducing NADH that could only be replaced by fructose derivatives, like sucrose and inulin (134). In addition, they found that the maximum mannitol production could be elevated by adding 33 mg/L $MnSO_4$ (135). It was also reported that *Leu. Pseudomesenteroides, L. brevis, Lactobacillus, Leuconostoc, Leu. mesenteroides*, etc. were all good natural mannitol producers,

and the ability for producing mannitol is tightly related to the MDH activity in cells (132). Different from heterologous fermentative LAB, which produce mannitol rely on reducing fructose, *L. lactis* and other homologous fermentative LAB (136)(137)(138) produce mannitol soley from glucose. *L. lactis* is the most studed homologous fermentatative LAB for mannitol production, and it will be discussed in the next section. In addition to LAB, yeast and fungi are also found to be good mannitol producers. Some yeast were found able to produce mannitol from pentose sugars and glycerol, like *Torulopsis mannitofaciens, Rhodotorula minuta* and *Candida magnoliae* (139)(140)(141). Song et al. reported a novel strain of *Candida magnoliae*, which could produce 209 g/L mannitol from fructose by using fed-batch culture (142). Some fungi were proved able to produce mannitol from glucose and other sugars, such as *Pirularia oryzae*, *Aspergillus candidus*, *Penicillium. Scabrosum*, where the last one could produce 43 g/L mannitol from sucrose (143)(144)(145).

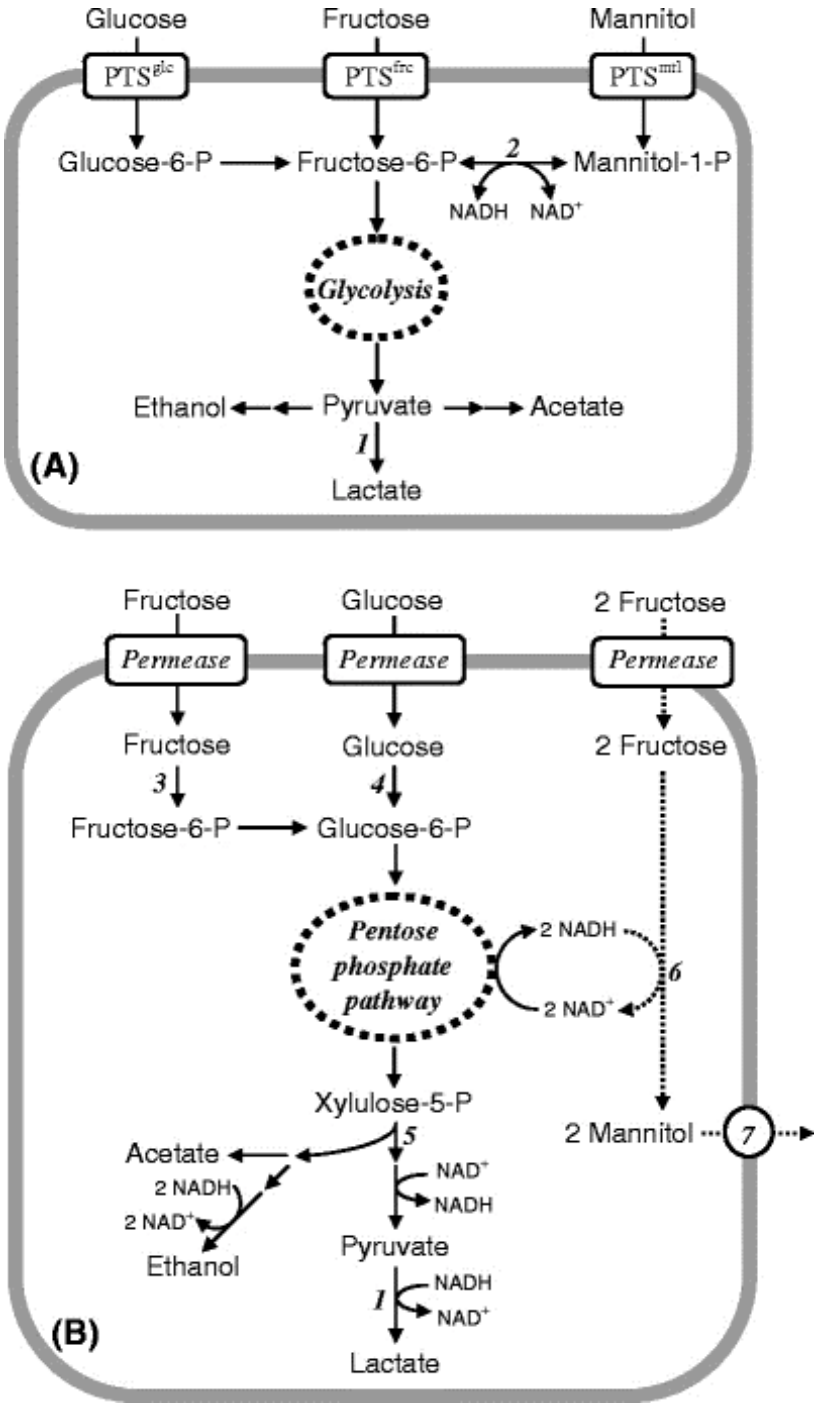


**Figure 1.8. Hexose metabolism in homofermentative (a) and heterofermentative LABs (b)**. PTS glc, PTS frc, and PTS mtl: glucose-specific, fructose-specific, and mannitol-specific phosphotransferase systems, respectively; 1 lactate dehydrogenase, 2 mannitol-1-phosphaste dehydrogenase, 3 fructokinase, 4 glucokinase, 5 phosphoketolase, 6 mannitol dehydrogenase, and 7 unknown. Solid arrows hexose assimilation pathways, dashed arrows fructose reduction to mannitol in glucose/fructose-grown heterofermentative LABs. Reprinted with permission from (124), Copyright (2009) Springer Nature.

### 1.6.3 Mannitol production in engineered *L. lactis*

Although there have been several achievements for producing mannitol from heterologous LAB and other microbes, most of them rely on the reaction of reducing fructose by MDH. Differently, homologous LAB produce mannitol base on reducing F6P, which means they could produce mannitol from all sugars that are metabolized into the glycolysis pathway (Figure 1.8). Considering *L. lactis* involves in many food industrial practices and equips with all the necessary enzymes for producing mannitol, it is promising to develop *L. lactis* as a mannitol-producing platform, though *L. lactis* is not a natural mannitol producer. The potential for using *L. lactis* as a mannitol producer is proposed by Neves et al. in 2002, after they detected intracellular M1P from an LDH deficient *L. lactis* strain, where the regeneration of intracellular $NAD^+$ was hampered (146). To date, mannitol production in *L. lactis* was only reported by using LDH deficient strains under anaerobic condition, which likely due to the hampered $NAD^+$ regeneration would force the cells rerouting the NADH flux to generate M1P from F6P. Instead of producing mannitol, *L. lactis* could naturally uptake mannitol by its PTS sugar transport system, and this should be considered before developing a mannitol-producing *L. lactis*. To avoid mannitol uptaking in *L. lactis*, inactivating mannitol specific EIIA or EIIB would be good ideas. After applying this strategy, Gaspar et al. first achieved mannitol production in *L. lactis* by using resting cells, with 32.8% yield from glucose, and with lactate, formate, acetate, ethanol, 2,3-butanediol, and acetoin as byproducts (147). In their works, intracellular M1PDH activity was also measured and it was shown to be around 2.5 U/mg protein, which was deduced to be the reason for the inefficient mannitol production in *L. lactis*. To enhance mannitol production, Wisselink et al. introduced *Lactobacillus plantarum mtlD* gene, which encodes M1PDH into a LDH-deficient strain, resulting in a 25% yield of mannitol produced from glucose in resting condition (148). In contrast, when using the same strain in growing condition, there was only 1% mannitol produced from glucose. Although MPDH and M1Pase activity were detected from cell extracts in both conditions. They proposed that the big difference between growing and non-growing cells on mannitol production is likely due to the following reasons: high F6P pool resulted from the low glycolytic flux, higher priority of NADH regeneration and lower ATP demand in resting condition. Interestingly, by introducing *Eimeria tenella mtlP*, which encodes M1Pase and by overexpressing *mtlD* together, it was possible to achieve 42-50% mannitol yield from glucose with growing LDH-deficient *L. lactis* (149)(70), suggesting an important role of M1Pase in mannitol production. However, solely overexpressing M1Pase or M1PDH would not result in mannitol production in growing

conditions. These results indicated a great potential of using *L. lactis* as a mannitol producer. Although the native enzyme related to M1Pase in *L. lactis* is uncharacterized, a strain with high M1Pase and M1PDH activity would be a great candidate for efficient mannitol production without foreign genes. However, little is known for how the relevant genes are regulated in *L. lactis* to date.

### 1.7 Objectives

Although there are several achievements for developing mannitol-producing *L. lactis*, most of the studies rely on introducing foreign genes and there are still lots of unknowns that limit the improvements in this research topic. As a homologous fermentative LAB, *L. lactis* could theoretically produce mannitol from carbon sources that undergo the central metabolism pathway. However, these relevant enzymes showed low activity and unclear how their coding genes are regulated in cells, for instance, it is even not clear that which gene is responsible for the M1Pase activity in *L. lactis* (149). Expressing foreign genes could easily bypass these puzzles, but the plasmid burden and the use of antibiotics would also leading to a hampered growth and lower biomass (70). Furthermore, such strains are not acceptable to use in food-relevant products, which would further limit their applications. Thus, the objectives of this study are to investigate the expression and regulation of the native genes related to mannitol production and developing food-grade mannitol producing strains by combining different strategies, such as laboratory evolution, genetic engineering, and fermentation optimization, etc.

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# Chapter 2. Laboratory evolution and NADH flux rewiring for enhancing mannitol production

This chapter is based on our work ‘‘Harnessing Adaptive Evolution to Achieve Superior Mannitol Production by *Lactococcus lactis* Using Its Native Metabolism’’, which has been published by *Journal of Agricultural and Food Chemistry*, 2020.

## 2.1 Abstract

Mannitol can be obtaind as a byproduct of certain heterolactic lactic acid bacteria, when grown on substrates containing fructose. *Lactococcus lactis*, a homolactic lactic acid bacterium, normally does not form mannitol, but can be persuaded into doing so by expressing certain foreign enzyme activities. In this study, we find that *L. lactis* has an inherent capacity to form mannitol from glucose. By adaptively evolving *L. lactis*, or derivatives blocked in $NAD^+$ regenerating pathways, we manage to accelerate growth on mannitol. When cells of the adapted strains are resuspended in buffer containing glucose, 4-58% of the glucose metabolized is converted into mannitol, in contrast to non-adapted strains. The highest conversion was obtained for a strain lacking all major $NAD^+$ regenerating pathways. Mannitol had an inhibitory effect on the conversion, which we speculated was due to the mannitol uptake system. After its inactivation, 60% of the glucose was converted into mannitol by cells suspended in glucose buffer, which is the highest yield reported for *L.lactis*.

**Keywords:** *L. lactis*; Mannitol; Adaptive evolution; NADH supply; ATP

## 2.2 Introduction

Mannitol, a six-carbon sugar polyol that exists naturally in many plants, especially in seaweeds, has many applications in the pharmaceutical, chemical and food industries. Clinically, mannitol is widely used in the management of cerebral oedema and other conditions with raised intracranial pressure (1),(2),(3),(4). In diabetic foods, due to its low glycemic index and is poor intestinal absorption, it is used as a sweetener, and since it is non-hygroscopic and does not promote dental decay it is commonly used in sweets(5).

Mannitol is mainly produced by chemical synthesis, usually from glucose/fructose mixtures, however, due to low yields, around 20%, alternative ways for producing mannitol have been sought after (6). In the last twenty years, numerous attempts have been made to produce mannitol by using heterofermentative LAB. Good yields and titers of mannitol have been obtained using different *Leuconostoc* and *Lactobacillus* species, and here fructose is reduced to mannitol using mannitol dehydrogenase (MDH) (7),(8). The main drawback of using these organisms for producing mannitol is that fructose-containing feedstocks are needed, which puts restraints on yields and cost (9).

*L. lactis* is a food-grade LAB (lactic acid bacterium) widely used in cheese production. As a homofermentative bacterium, it converts up to 95% of the sugar it metabolizes to lactate, when

grown anaerobically. In the past decade, it has been demonstrated that *L.lactis* can generate mannitol from fructose-6-phosphate (F6P), a central metabolite in glycolysis (10),(11). Using *L. lactis* to produce mannitol from other sugars besides fructose at high yield and titer, could help lower the cost of mannitol production. A challenge, however, is that the wild-type *L. lactis* strains only produce mannitol in low amounts. Intracellular mannitol can only be detected in lactate dehydrogenase (Ldh) deficient strains, and is detectable in culture medium only when using resting cells (non-growing cells) (10). In resting conditions, the glucose to mannitol conversion ratio obtained for an Ldh deficient strain is around 25-33% (12),(13). After complementing with mannitol 1-phosphate phosphatase (*mtlP*) from *Eimeria tenella*, and mannitol-1-phosphate 5-dehydrogenase (*mtlD*) from *Lactobacillus plantarum*, it was shown that an Ldh deficient strain could produce mannitol in growing conditions with mannitol/glucose (M/G) ratios of 42-50%, suggesting key roles of these enzymes in mannitol production (14),(15).

Mannitol-1-phosphate (M1P) formation from F6P is catalyzed by MtlD, and consumes 1 NADH (Figure 2.1). The observation that mannitol only can be produced in an Ldh deficient strains, shows how important a sufficient NADH supply is for mannitol production. Based on redox considerations, in *L. lactis*, the theoretical maximum conversion ratio from glucose should be 66.7%. There is thus still a large potential for increasing the M/G ratio in *L. lactis*.

In this study, rather than relying on expression of foreign genes, we use adaptive laboratory evolution to achieve mannitol production in *L. lactis*. We increase mannitol yield by blocking metabolic pathways consuming NADH and carry out experiments with both resting and growing cells. Finally, we obtain a strain, which can convert more than 60% of the glucose consumed into mannitol, which is the highest mannitol conversion ratio reported for *L. lactis* to our knowledge.

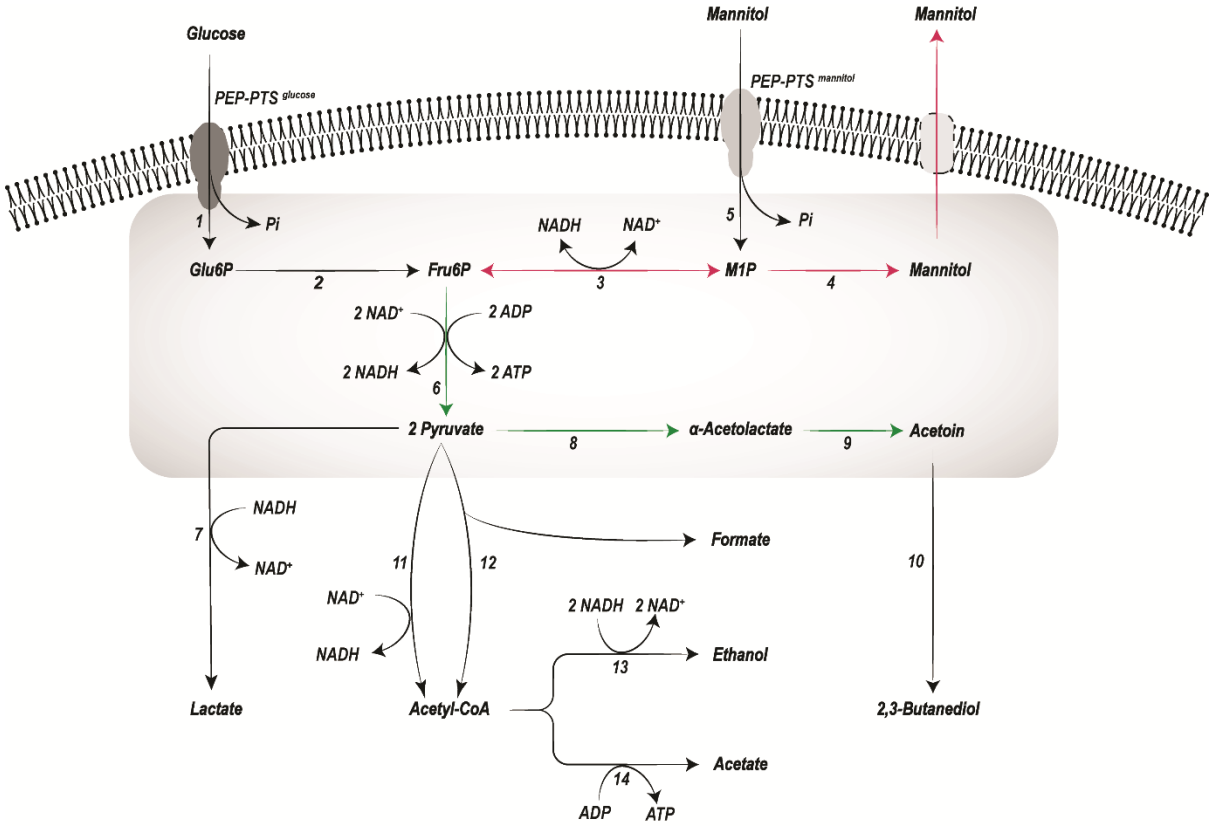


**Figure 2.1. Illustration showing the central sugar metabolism and mannitol synthesis pathway in *L. lactis*.** NADH formation and consumption as well as ATP formation is indicated. The following enzymes are involved: 1. phosphoenolpyruvate (PEP)-dependent mannitol phosphotransferase system (PTS); 2. phosphoglucoisomerase; 3. mannitol-1-phosphate 5-dehydrogenase; 4. unknown phosphatase in *L. lactis*; 5. mannitol-specific PTS system; 6. enzymes including 6-phosphofructokinase, fructose 1,6-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase and pyruvate kinase; 7. lactate dehydrogenase; 8. α-acetolactate synthase; 9. α-acetolactate decarboxylase; 10. 2,3-butanediol dehydrogenase; 11. pyruvate dehydrogenase complex; 12. pyruvate-formate lyase; 13. acetaldehyde dehydrogenase and alcohol dehydrogenase; 14. phosphate acetyltransferase and acetate kinase.

## 2.3 Methods

### Bacterial strains and growth conditions

*Lactococcus lactis* subsp. *cremoris* MG1363 and its derivatives CS4099, CS4363, Ace001, AceF were used in this study (described in Table 2.1). All these strains are plasmid-free. *L. lactis* strains were cultured in M17 broth supplemented with 1% glucose (Sigma-Aldrich) unless indicated otherwise. For laboratory adaptation, all strains used, except AceF, were

cultured aerobically in M17 supplemented with 0.5% mannitol and 2.5 μg/ml hemin. After reaching stationary phase, 1 ml was transferred to 9 ml fresh medium. For measuring the growth rate of different strains, cells were precultured until reaching the early exponential phase, then transferred into startup medium at a cell density of $OD_{600}$ = 0.02, and finally cultured aerobically in M17 at 30°C. Cell density was measured using a spectrophotometer (Shimadzu) at 600 nm. Growth rates were calculated as described by Friedrich (16).

**Preparation and cultivation of resting cells**

For preparing resting cells, *L. lactis* was pre-cultured in M17 supplemented with 0.5% glucose and 2.5 μg/ml hemin under aerobic conditions. The cells were harvested in the early exponential phase, washed two times using PBS (pH = 7.0), and resuspended in PBS supplemented with 2% glucose at an $OD_{600}$ around 15.0. The suspensions were incubated at 30°C under anaerobic conditions and sampled at specific time points.

**Quantifying metabolites by high-performance liquid chromatography (HPLC)**

For quantifying the sugars and metabolites produced, an HPLC (Dionex) equipped with a Shodex RI-101 refractive index detector (Showa Denko K.) and a BioRad Aminex HPX-87H column (Bio-Rad) was used. The mobile phase was water containing 5 mM $H_2SO_4$. The temperature of the column oven was set to 60°C and a flow speed of 0.5 ml/min was used. The samples for HPLC analysis were filtered using 0.22 μM filters (Labsolute) immediately after sampling, and were stored at -20°C until use.

**Molecular techniques**

Electro-component *L. lactis* cells were prepared in GM17 with 2% glycine and 0.25 M sucrose, then harvested at the pre-exponential phase. DreamTaq Hot Start DNA Polymerase (Thermofisher Scientific) was used for PCR. For plasmid extraction from *L. lactis*, cells were first incubated with 20 mg/ml lysozyme (Fluka, 7000U/mg) solution at 37°C for 2 hours, and subsequently a Zyppy plasmid mini-prep kit (Zymo Research) was used for extracting the plasmids. DNA sequencing was performed by Macrogen, South Korea.

**Construction of strains and plasmids**

All strains and plasmids used in this work are listed in Table 2.1. To delete the gene *mtlF*, a plasmid with a thermosensitive replicon, $pG^+$host8 (17), was used. gBlocks containing in total approximately 2400bp of DNA upstream and downstream the region to be deleted were

synthesized by IDT. Both pG$^+$host8 and gBlocks were digested with restriction enzymes (Thermofisher Scientific) BamHI (GGATCC) and XbaI (TCTGA) and ligated together using T4 ligase (Thermofisher Scientific). The ligated plasmid, designated pKmtlF, was introduced by electroporation into *E. coli* MC1000(18). To obtain the desired gene deletion in *L. lactis*, the approach described briefly below was used: pKmtlF was transferred into *L. lactis* by electroporation at 2kV. After short incubation at 28°C, transformants were spread on GM17 plates, containing 5 μg/ml tetracycline and incubated at 37°C. After two days of incubation, the presence of big colonies indicated recombination of the plasmid into the chromosome (crossing-in) had taken place. Large colonies were then, after restreaking at 37°C, streaked on new plates without tetracycline and incubated at 28°C to allow for crossing-out of the plasmid. Big colonies at 28°C indicated that a crossing-out event had taken place, and this was verified by streaking the strains on plates with/without tetracycline at 37°C and by using PCR.

**Table 2.1. Strains and plasmids used in this work**

| **Strain and plasmid** | **Relevant genotype** | **Source or reference** |
|---|---|---|
| ***L.lactis* strains** | | |
| **MG1363** | Wild-type *L. lactis* subsp. *cremoris* | (19) |
| **MG363-M** | MG1363 adapted on mannitol | This work |
| **CS4099** | MG1363 *Δ*$^3$*ldh* (*Δldh, ΔldhB and ΔldhX*) | (20) |
| **CS4099-M** | CS4099 adapted on mannitol | This work |
| **CS4363** | MG1363 *Δ*$^3$*ldh*, *Δpta*, *ΔadhE* | (20) |
| **CS4363-M** | CS4363 adapted on mannitol | This work |
| **Ace001** | MG1363 *Δ*$^3$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA* | (21) |
| **Ace001-M** | Ace001 adapted on mannitol | This work |
| **AceF** | Ace001-M *ΔmtlF* | This work |
| **MC1000** | *E.coli* cloning host | (18) |
| **Plasmid** | | |
| **pG$^+$host8** | *E. coli*/*L. lactis* shuttle vector, Tet$^R$, thermosensitive replicon | (17) |
| **pKmtlF** | Used for knocking out *mtlF* | This work |

### Whole genome sequencing

Genome of MG1363, MG1363-M, CS4099, CS4099-M, CS4363, CS4363-M, Ace001, Ace001-M were extracted by Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research) according to manufacturer's manual. Whole genome sequencing was performed by short insert fragment library preparation and PE150 sequencing with 1Gb clean data per sample (BGISEQ). Sequencing data analysis, genome mapping and variations finding were performed by the software in Geneious Prime 2019. The program BBDuk was used for trimming by removing the adapters at the left end, cutting sequences with quality less than 15.0 at both ends and discarding reads shorter than 50bp. Program Bowtie2 was then used for aligning the trimmed reads to the reference sequence (*L. lactis* MG1363, Genbank Accession number: NC009004) by default settings. SNVs (single nucleotide variations) were found by meeting the requirements of variant frequency no less than 90% and strand bias not exceeding 75%. Finally, the found SNVs were compared between all mannitol adaptive strains and their parental strains.

## 2.4 Results

### 2.4.1 Enhancing mannitol production in resting *L.lactis*

### Accelerated growth on mannitol after adaptive laboratory evolution

*L. lactis* can grow slowly on mannitol, which demonstrates that all the enzymes needed for mannitol catabolism are present, but perhaps some of these are poorly expressed. In an attempt to overcome the putative bottleneck, we decided to carry out an adaptive laboratory evolution experiment to obtain mutants with accelerated growth on mannitol. *L. lactis* MG1363 was grown aerobically in M17 containing mannitol as the sole carbon source for more than 2 months. Aerated conditions were chosen since mannitol is more reduced than glucose and aeration is known to facilitate more efficient regeneration of $NAD^+$. In the course of the evolution the faster growing isolate MG1363-M was obtained and its growth characterized. As shown in Figure 2.2, MG1363 grows slowly ($\mu_{max}$ = 0.067) on mannitol and can reach stationary phase after 30 hours. In M17 medium, a two-phased growth pattern was observed due to the presence of additional metabolizable sugars (without added sugars a cell density of around 0.6 can be reached in M17). MG1363-M, however, grew much faster than MG1363 in mannitol ($\mu_{max}$ = 0.472), although slower than on glucose ($\mu_{max}$ = 0.665). These results demonstrated that it was possible to increase growth rate of *L. lactis* on mannitol.

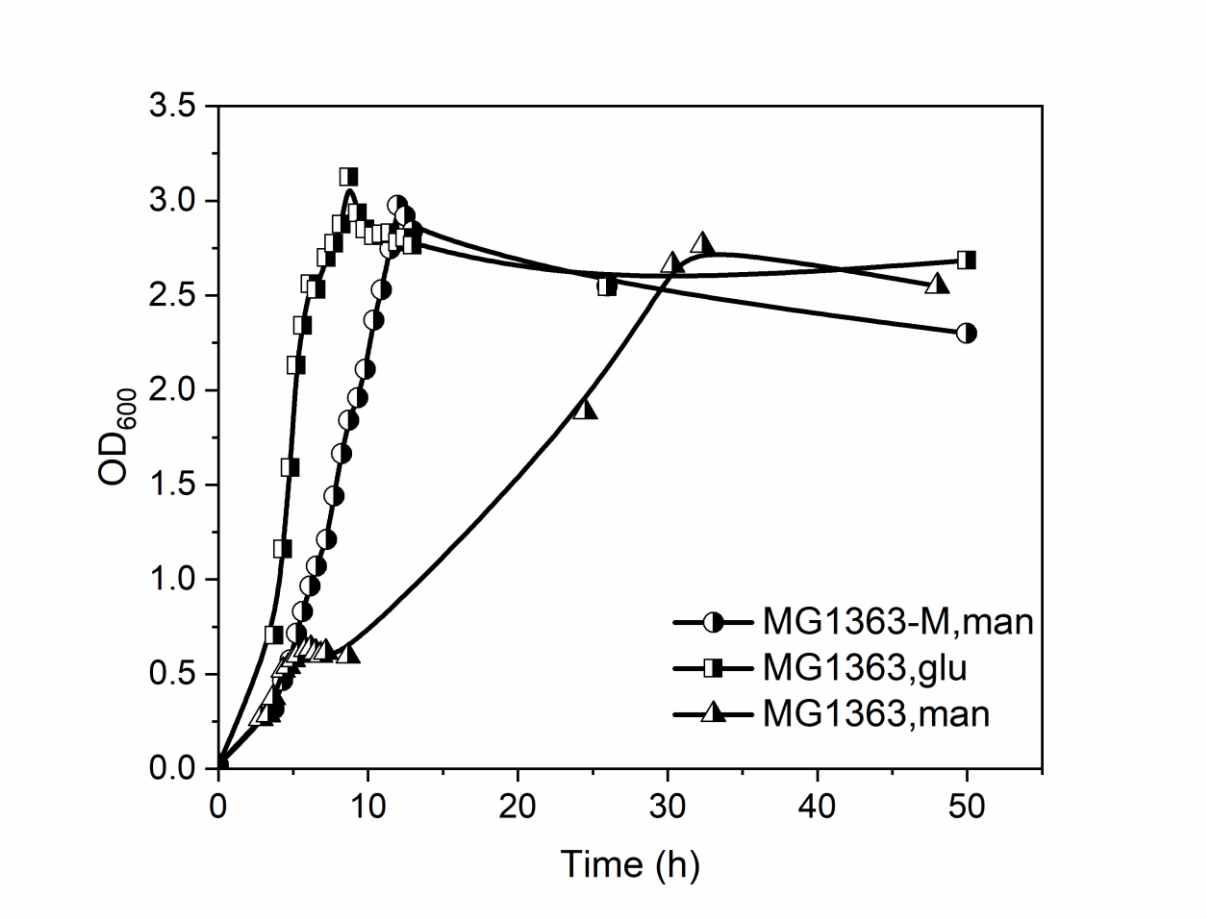


**Figure 2.2. Growth performance of MG1363 and MG1363-M on mannitol supplemented M17 broth.** Growth of MG1363 and MG1363-M in M17 supplemented with 0.5% of the indicated sugar. Rectangle: MG1363 grown on glucose; circle: MG1363 grown on mannitol; triangle: MG1363-M grown on mannitol.

## Adaptation to fast growth on mannitol leads to mannitol and acetoin formation from glucose in resting cells under static conditions

MG1363-M is clearly better at metabolizing mannitol, and since the mannitol metabolism in *L. lactis* has been reported to be reversible, we suspected that the mannitol adapted strain MG1363-M might also be better at producing mannitol from glucose. We decided to test the adapted strain under resting and static conditions to determine whether mannitol could be produced from glucose. Under static conditions, no active aeration takes place, however, slow diffusion of atmospheric oxygen into the cell suspension is possible, thus allowing for the NADH oxidase to slowly convert NADH into $NAD^+$. As shown in Figure 2.3, MG1363-M indeed generated mannitol from glucose, and approximately 4% of the glucose was channeled

to mannitol. In contrast, the wild-type strain MG1363 only produced lactate. We also tested whether mannitol could be formed by MG1363-M suspension when aerated, however, as expected, no mannitol was detected, indicating that the NADH supply under these conditions was insufficient, probably due to NADH consumption by the NADH oxidase (*noxE*) in the presence of oxygen.

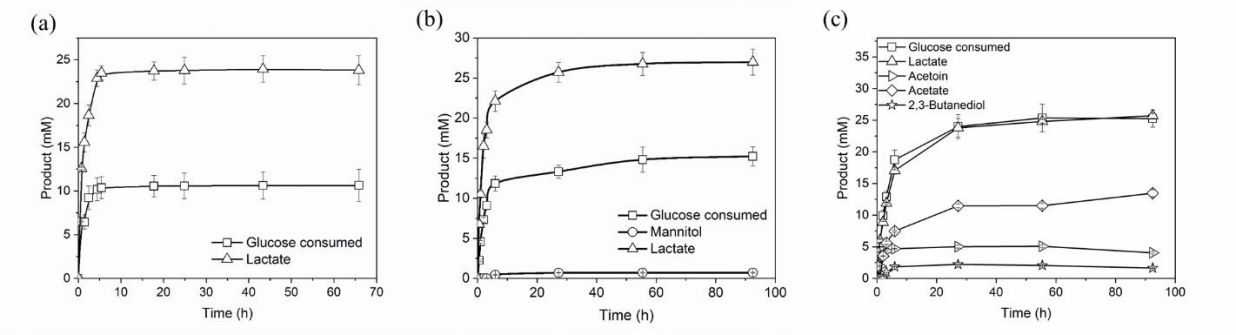


**Figure 2.3. Mannitol production in resting cells of MG1363 and MG1363-M**. (a) Glucose consumption and products generated by MG1363 under static (no active aeration, slow diffusion of oxygen possible) conditions; (b) glucose consumption and products produced by MG1363-M under static conditions; (c) glucose consumption and products produced by MG1363-M under aerobic conditions. Experiments were conducted in duplicates and error bars indicate standard deviations. Cells ($OD_{600}$=15.0) were suspended with PBS buffer (pH=7.0) supplemented with 2% glucose.

### 2.4.2 Metabolic engineering for narrowing NADH flux in *L.lactis*

#### Increasing the NADH supply is beneficial for mannitol production

To enhance the NADH supply, we decided to explore an MG1363 derivative lacking lactate dehydrogenase activity (CS4099), and a derivative that furthermore lacks the alcohol dehydrogenase and the phosphotransacetylase (CS4363). Both strains were adapted for fast growth on mannitol and two adapted derivatives, named CS4099-M and CS4363-M, were isolated and further characterized in resting cells experiments. No mannitol was detected from both of the non-adapted strains CS4099 and CS4364, however, in contrast, the adapted strains formed large amounts of mannitol. As shown in Figure 2.4, CS4099-M converted approximately 21% of the glucose to mannitol, with lactate, 2,3-butanediol and acetate as by-products. CS4363-M had more than doubled the mannitol conversion ratio to 43% with 2,3-butanediol as the sole byproduct (>50%). Under aerated conditions, CS4099-M produced

lactate, acetoin, 2,3-butanediol and acetate without any mannitol being detected. For CS4363-M, nearly 90% of the by-product formed was acetoin. Compared with cells incubated anaerobically, the dramatically decreased yield of 2,3-butanediol indicated that $NAD^+$ could be regenerated efficiently by the NADH oxidase.

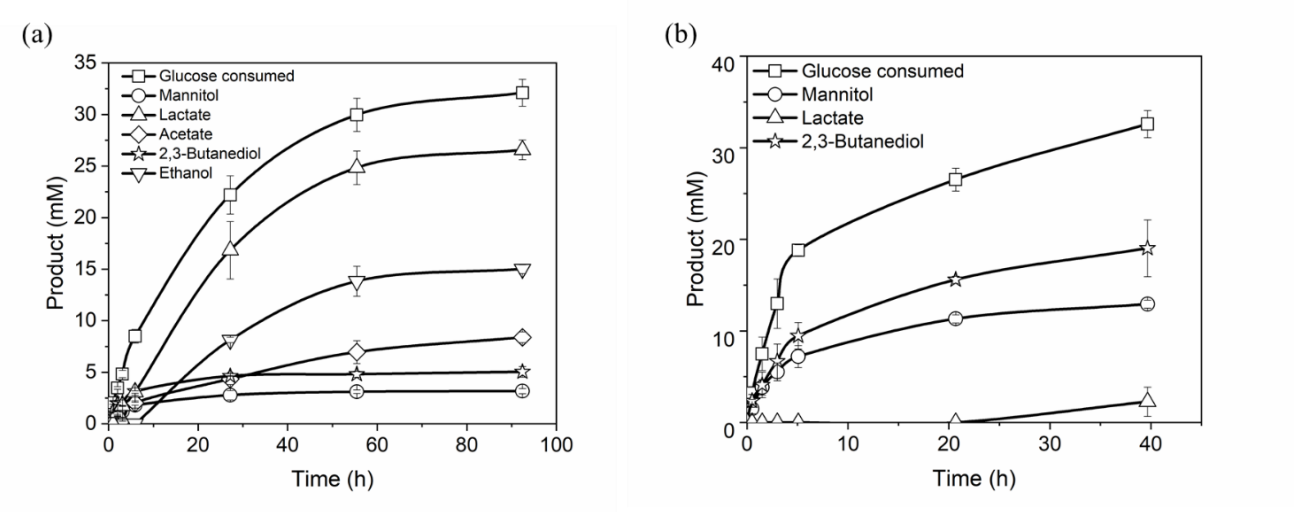


**Figure 2.4. Mannitol production by resting cell cultures of CS4099-M and CS4363-M.** (a) Glucose consumption and products produced by resting cells of CS4099-M ; (b) glucose consumption and products produced by resting cells of CS4363-M. Static conditions are used (no active aeration, slow diffusion of oxygen possible). Experiments were conducted in duplicate and error bars indicate standard deviations. Cells ($OD_{600}$=15.0) were suspended with PBS buffer (pH=7.0) supplemented with 2% glucose.

## Channeling more NADH into mannitol production by blocking 2,3-butanediol formation

2,3-Butanediol is formed from acetoin by butanediol dehydrogenase (encoded by *butBA*), which consumes one NADH. In order to get rid of the by-product 2,3-butanediol and thereby further boost the NADH supply for mannitol production, we relied on *L. lactis* Ace001, a strain that is derived from CS4363 by deleting *butBA*. In Ace001, mannitol production is the only way in which $NAD^+$ can be regenerated, and for this reason Ace001 grows poorly under static conditions. In resting conditions, Ace001 formed only small amounts of acetoin, most likely due to the NADH oxidase and oxygen diffusing into the cell suspension, and no mannitol was detected. After adaptation on mannitol for nearly one month, the Ace001-M was obtained. As shown in Figure 2.5, the conversion ratio for Ace001-M increased to 58% and the only other

by-product observed was acetoin. Intriguingly, a lactate peak was detected after 20 hours of incubation, which coincided with a decrease in mannitol concentration and the mannitol conversion ratio therefore decreased to less than 40%. Another important observation was that mannitol accumulation appeared to inhibit mannitol production (data not shown). For this reason, we decided to delete *mtlF*, encoding the mannitol specific soluble PTS component (IIA) in Ace001-M.

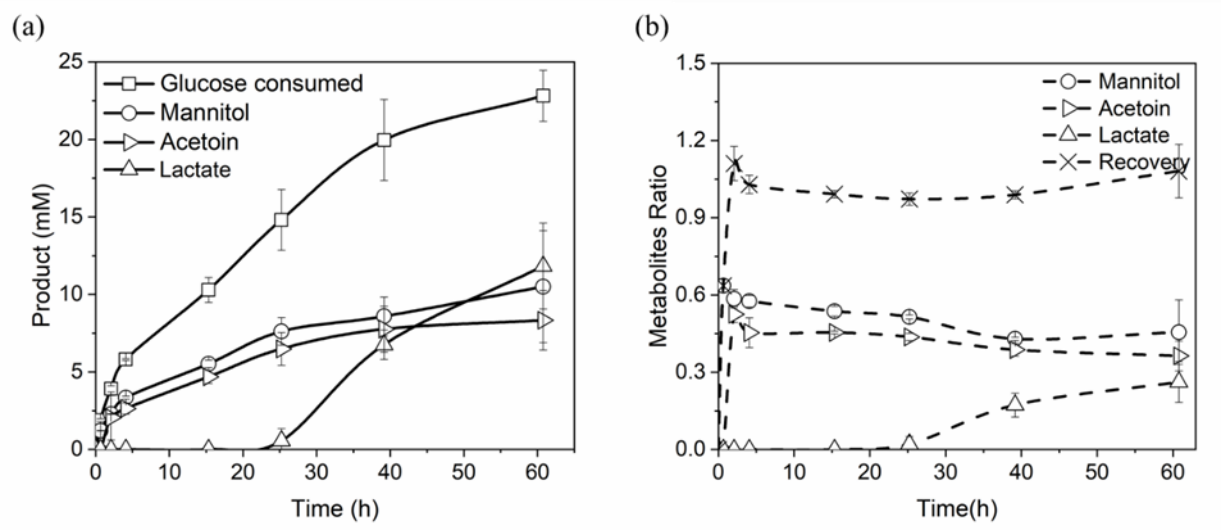


**Figure 2.5. Mannitol production by resting cells of Ace001-M.** (a) Glucose consumption and products produced from Ace001-M; (b) the conversion ratio of all metabolites from glucose and recovery of glucose in Ace001-M. Static conditions are used (no active aeration, slow diffusion of oxygen possible). Experiments were conducted in duplicate and error bars indicate standard deviations. Cells ($OD_{600}$=15.0) were suspended with PBS buffer (pH=7.0) supplemented with 2% glucose.

### Knocking-out mannitol uptake further stimulates mannitol production

A 302bp DNA sequence that included the promoter of *mtlF* gene was deleted in Ace001-M as described in the Materials & Methods section, and the strain obtained was designated AceF. Without mannitol transport activity, AceF grew poorly in M17 supplemented with mannitol. AceF, however, could still convert glucose to mannitol and mannitol could be transported out of the cell. As shown in Figure 2.6, AceF had a mannitol conversion ratio of 60%, which is the highest M/G ratio we achieved. In addition, unlike Ace001-M, when a high concentration of mannitol was present in the cell suspension, there was no inhibitory effect of mannitol on mannitol production. Interestingly, after deleting *mtlF*, lactate was no longer formed.

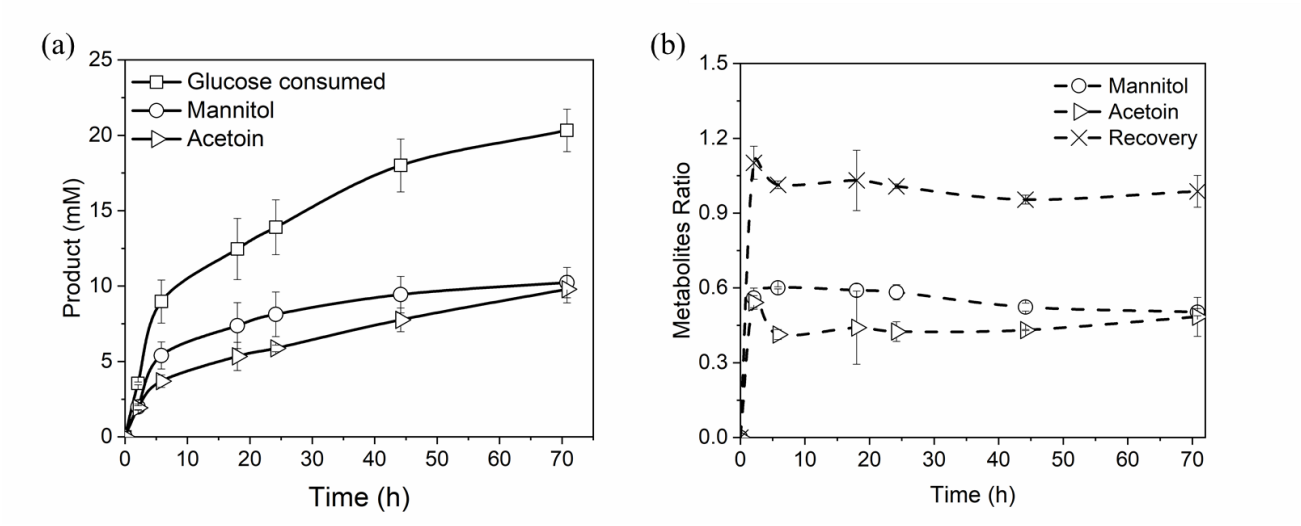


**Figure 2.6. Mannitol formation by resting cells of AceF.** (a) Glucose consumption and byproducts produced by AceF; (b) the conversion ratio of all metabolites from glucose and recovery of glucose in AceF. Static conditions are used. Experiments were conducted in duplicate and error bars indicate standard deviations. Cells ($OD_{600}$=15.0) were suspended with PBS buffer (pH=7.0) supplemented with 2% glucose.

**Table 2.2. On overview of by-product formation for all strains used in this study in resting cells experiments**

| Resting cells | Glucose | Mannitol | Lactate | Acetoin | Acetate | 2,3-BDO | Ethanol | Recovery |
|---|---|---|---|---|---|---|---|---|
| MG1363 | 1 | nd | 1.07±0.08 | nd | nd | nd | nd | 1.07±0.08 |
| MG1363-M | 1 | 0.04±0.00 | 0.95±0.02 | nd | nd | nd | nd | 0.99±0.02 |
| MG1363-M, $O_2$ | 1 | nd | 0.46±0.03 | 0.34±0.01 | 0.22±0.01 | 0.07±0.01 | nd | 0.98±0.05 |
| CS4099 | 1 | nd | nd | nd | nd | 0.53±0.06 | 0.36±0.04 | 0.89±0.02 |
| CS4099-M | 1 | 0.21±0.02 | 0.18±0.01 | nd | 0.12±0.00 | 0.37±0.01 | nd | 0.88±0.03 |
| CS4099-M, $O_2$ | 1 | nd | 0.13±0.02 | 0.46±0.04 | 0.22±0.02 | 0.17±0.02 | nd | 0.98±0.03 |
| CS4363 | 1 | nd | nd | nd | nd | 0.55±0.05 | nd | 0.55±0.05 |
| CS4363-M | 1 | 0.43±0.01 | nd | nd | nd | 0.51±0.04 | nd | 0.94±0.02 |
| CS4363, $O_2$ | 1 | nd | nd | 0.93±0.01 | nd | 0.04±0.00 | nd | 0.97±0.00 |
| CS4363-M, $O_2$ | 1 | nd | nd | 0.88±0.01 | nd | nd | nd | 0.88±0.01 |
| Ace001 | 1 | nd | nd | 0.82±0.04 | nd | nd | nd | 0.82±0.04 |
| Ace001-M | 1 | 0.58±0.02 | nd | 0.45±0.06 | nd | nd | nd | 1.03±0.04 |
| AceF | 1 | 0.60±0.00 | nd | 0.41±0.02 | nd | nd | nd | 1.01±0.02 |

Shown are average values ± standard deviations from at least two independent growths.The abbreviation nd indicates “not detectable” from HPLC results. All data shown here were collected from samples taken prior to 15-hours of cultivation.

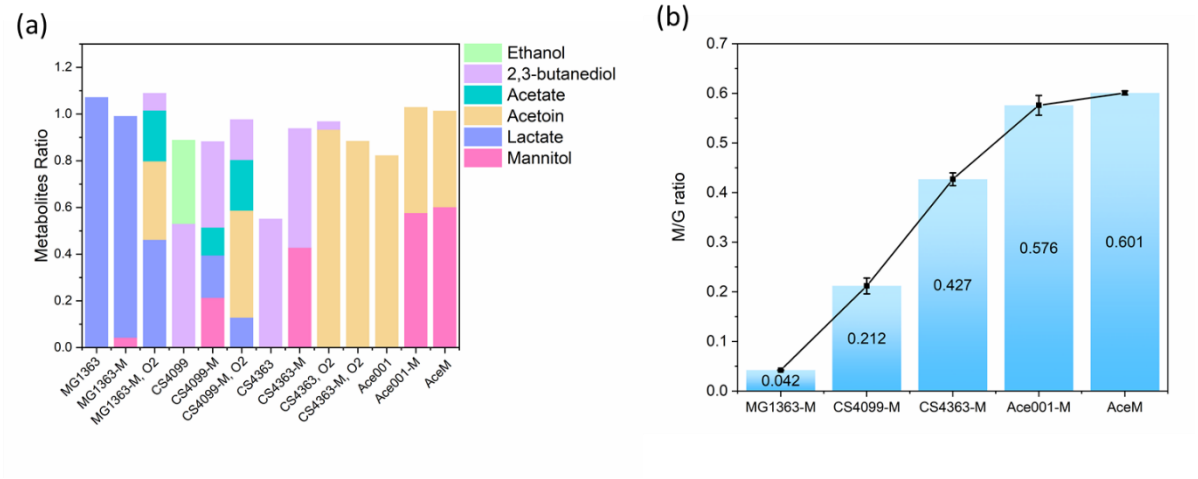


**Figure 2.7. An overview of the byproducts formed by the different strains studied in resting cells experiments and the corresponding mannitol/glucose conversion ratios.** (a) comparison of product ratios obtained for different strains; (b) Mannitol yield for strains adapted on mannitol. $O_2$ indicates aeration. All data shown were collected from samples withdrawn prior to 15-hours of cultivation.

### Mannitol production by the adapted strains under static conditions supporting growth

We also characterized the adapted strains in terms of mannitol production under static conditions (no active aeration, slow stirring) supporting growth, i.e. in M17 broth supplemented with glucose. Mannitol was not formed by MG1363-M, CS4099-M and CS4363-M. In contrast 2-4 mM of mannitol could be detected for Ace001-M and 4-8mM mannitol for AceF (data not shown). However, both Ace001-M and AceF grew poorly under static conditions.

### Sequencing reveals no mutations in the gene encoding mannitol-phosphate dehydrogenase

As shown in Table 2.2, mannitol formation was only detected for mannitol adapted strains. In *L. lactis*, F6P is reduced by mannitol-1-phosphate 5-dehydrogenase (*mtlD*) to M1P, and subsequently M1P is dephosphorylated by an unidentified phosphatase. Due to its involvement in both mannitol catabolism and mannitol synthesis, we speculated that the mannitol-1-phosphate dehydrogenase locus could have mutated in the mannitol adapted strains, thereby resulting in a higher expression level or perhaps a more active enzyme. We therefore sequenced *mtlD* and its 3' and 5' flanking sequences in AceF and Ace001. No mutations, however, were

revealed, and we could conclude that there was other reasons for the increased mannitol production capacity of the evolved strains.

### 2.4.3 Genome sequencing reveals the key SNPs for superior mannitol production in *L.lactis*

In order to find the underlying explanation for the enhanced growth on mannitol, as well as the superior ability to transform glucose into mannitol, for the adapted strains, we sequenced their genomes as well as that of the parent strains. As shown in Table 2.3, we found, in total, 16 single-nucleotide variations (SNVs) in all mannitol adapted strains, whereof 13 were unique, including 8 in protein coding regions and 3 in intergenic regions. Interestingly, all 4 mannitol adapted strains had mutations upstream of *mtlA*, and 3 of them (except CS4099-M) had mutations in the *mtlF* coding region. In addition to these mutations, we also detected SNVs in genes encoding an amino acid permease, an SpaH/EbpB family LPXTG-anchored major pilin, a type IV secretory system conjugative DNA transfer family protein, a Redox-sensing transcriptional repressor, an SGNH/GDSL hydrolase family protein, an Fe-S cluster assembly protein SufB and finally in the gene encoding an Acetyltransferase. All of the SNVs in gene CDS region leading to changes in amino acids or frameshift. And two more SNVs in intergenic regions were also detected. One near the gene encoding the NADH oxidase, and another between genes encoding an Endoglucanase and Polysacchride deacetylase family protein.

**Table 2.3. Single-nucleotide variations occurred after mannitol adaptation in different strains**

| Strains | RP[a] | Coverage | Region | NC[b] | AAC[c] | Protein id | Product or (distantance from CDS) neared product |
|---|---|---|---|---|---|---|---|
| MG1363-M | 26455 | 509 | Intergenic | C > T | | | (-39bp) PTS mannitol transporter subunit IIBC |
| MG1363-M | 30716 | 656 | Gene CDS | C > T | A > V | WP_011834136.1 | PTS mannitol transporter subunit IIA |
| MG1363-M | 369233 | 550 | Gene CDS | G > A | P > L | WP_011834429.1 | amino acid permease |
| MG1363-M | 1132975 | 199 | Intergenic | A > G | | | (+103) Endoglucanase and (-149) Polysacchride deacetylase family protein |
| MG1363-M | 1779597 | 421 | Gene CDS | C > A | L > F | WP_011835577.1 | SpaH/EbpB family LPXTG-anchored major pilin |
| CS4099-M | 26455 | 522 | Intergenic | C > T | | | (-39bp) PTS mannitol transporter subunit IIBC |
| CS4099-M | 1357615 | 515 | Gene CDS | C > T | E > K | WP_011835247.1 | type IV secretory system conjugative DNA transfer family protein |
| CS4363-M | 26448 | 495 | Intergenic | G > T | | | (-46bp) PTS mannitol transporter subunit IIBC |
| CS4363-M | 30518 | 845 | Gene CDS | G > A | G > D | WP_011834136.1 | PTS mannitol transporter subunit IIA |
| CS4363-M | 1488149 | 633 | Gene CDS | -T | Frameshift | WP_011835356.1 | Redox-sensing transcriptional repressor |
| CS4363-M | 1582199 | 393 | Gene CDS | T > G | E > A | WP_011835437.1 | SGNH/GDSL hydrolase family protein |
| Ace001-M | 26455 | 506 | Intergenic | C > T | | | (-39bp) PTS mannitol transporter subunit IIBC |
| Ace001-M | 30716 | 775 | Gene CDS | C > T | A > V | WP_011834136.1 | PTS mannitol transporter subunit IIA |
| Ace001-M | 401743 | 615 | Intergenic | C > T | | | (-79bp) NADH oxidase |
| Ace001-M | 1951535 | 663 | Gene CDS | C > A | V > F | WP_004255207.1 | Fe-S cluster assembly protein SufB |
| Ace001-M | 2333009 | 551 | Gene CDS | C > A | D > Y | WP_011836032.1 | Acetyltransferase |

[a] Reference position, location is based on the genome sequence of *L. lactis* MG1363 in Genbank (Accession number: NC009004). [b] Nucleotide change. [c] Amino acid change.

## 2.5 Discussion

### Mannitol adaptation both facilitate mannitol uptake and mannitol production

The reduction of F6P to mannitol is catalyzed by mannitol-1-phosphate dehydrogenase (*M1PDH*), which is encoded by *mtlD*. Since the M1PDH activity in *L. lactis* is very low, different approaches have been used to obtain mannitol production in *L. lactis*. One research group managed to enhance mannitol production by over-expressing *mtlD* from *Lactobacillus plantarum* in a lactate deficient *L. lactis* strain, and achieved 25% mannitol conversion yield with resting cells (12). Gaspar et al. over-expressed native *mtlD* and *Eimeria tenella mtlP* in *L. lactis*, and achieved a 42% mannitol yield with growing cells, which demonstrates the importance of *mtlP* in mannitol production (14). Neves et al. detected M1PDH activity in *L. lactis* when it was grown on mannitol (11), and this inspired us to carry out laboratory adaptative evolution in an attempt to enhance the *M1PDH* activity. We found that *L. lactis* could indeed metabolize mannitol better after the evolution, and also that resting cells could convert glucose into mannitol. We expected that the change in behavior was linked to changed expression of the *M1PDH*, however, sequencing did not reveal any mutations in the *mtlD* gene or in its vicinity. In other studies where *mtlD* was overexpressed, mannitol production in resting cells was observed, which suggests that this enzyme indeed is important (12). To help find the underlying cause for the observed behavior, we performed full genome sequencing. Our sequencing data identified, in total, 11 SNV's in the mannitol adapted strains, and surprisingly one locus was mutated in all 4 adapted strains and another in three of the adapted strains. One of these were *mtlF*, where the mutations occurred in the CDS region, leading to changes in amino acids, G54D and A120V respectively. *L. lactis* takes up mannitol by a phosphoenolpyruvate (PEP)-dependent mannitol phosphotransferase system (PTS)(22),(23),(24),(25). In general, PEP-PTS has two general components, enzyme I (EI) and HPr, and several sugar-specific enzyme IIs (EIIs) complex, consisting of EIIABC proteins(26),(27). In *L. lactis*, *mtlF* encodes the mannitol specific EIIA of the mannitol PEP-PTS, which is responsible for transferring the phosphoryl goup from Hpr to the mannitol specific EIIBC(28),(29). The identified SNVs in *mtlF* CDS region indicates a change in EIIA protein structure that could facilitate phosphoryl group transfer between Hpr and EIIBC in the mannitol adapted strains. Other interesting mutations were found upstream *mtlA*, -46 and -39 bp's from first codon respectively. Considering that this region most likely contains the promoter of *mtlA*, the SNVs might change the expression level of *mtlA* in mannitol adapted

strains. *mtlA* encodes the mannitol specific EIIBC, which is responsible for transferring the phosphoryl group from EIIA to mannitol and this component also constitutes the transmembrane channel through which mannitol enters/leaves the cell (28),(29). It is likely that that EIIBC is merely over-expressed in the adapted strains, and that this increases mannitol uptake during growth and excretion when cells are suspended in glucose containing medium. Other SNVs may also be involved in the accelerated growth observed on mannitol after adaptation. The impaired growth observed for the non-adapted strains on mannitol could be alleviated in different ways. SNV found in the gene encoding an amino acid permease could facilitate uptake of nutrients from the medium and protect cells from sugar starvation. SNVs in the gene encoding a redox-sensing transcriptional repressor and in the vicinity of the gene encoding NADH oxidase may help cells adapt to a high $NADH/NAD^+$ condition. However, the effect of these SNVs remains to be evaluated in our future studies. We found that the mannitol adapted strains also were more efficient at generating mannitol from glucose. In 3 out of 4 mannitol adapted strains, mutations were found in *mtlF* and all adapted strains had mutations in *mtlA*. Taking into consideration that mannitol production was improved after deleting *mtlF*, it is likely that the SNV's observed in *mtlF* lead to reduced expression of this PTS component, even though that may appear to conflict with the improved growth phenotype observed on mannitol. It is, however, a well-known phenomenon that excessive accumulation of sugar phosphates could lead to hampered growth (30), and it is possible that reduced expression of *mtlF* could compensate for such an effect.

**A high NADH supply is key to obtaining a high mannitol yield**

In *L. lactis*, two-steps are needed for converting F6P into mannitol. F6P is first reduced into mannitol-1-phosphate by M1PDH, which is then dephosphorylated by an unknown sugar phosphatase (SP) into mannitol. From glucose one NADH and one ATP are consumed in the process. For the wild-type MG1363, it has been demonstrated that a lactate dehydrogenase deficient strain has higher intracellular M1PDH and SP enzyme activity, but despite the presence of 90 mM of intracellular mannitol, mannitol is not secreted (10). When the lactate dehydrogenase is disrupted, the main sink for NADH is gone, and the $NADH/NAD^+$ ratio increases (31),(32), and in such mutants the NADH is oxidized in a different way which leads to formation of ethanol, 2,3-butanediol and other products(33). In our study we also observed this for our mannitol adapted lactate dehydrogenase deficient strain, but not for the non-adapted strain, and the latter concurs with previous research (10). To achieve high mannitol production in a lactate dehydrogenase deficient strain, Wisselink et al. found that it was necessary to

overexpress *mtlD*, and after doing so managed to achieve 25% conversion of glucose into mannitol in resting cells (34). To further enhance mannitol production we eliminated ethanol and 2,3-butanediol formation and achieved a 58% conversion. Our results clearly illustrate the importance of a sufficient NADH supply for high yield mannitol production. We also demonstrated that no mannitol was produced under aerobic conditions, where the NADH oxidase regenerates $NAD^+$.

**Mannitol uptake has a negative effect on mannitol production**

*L. lactis* is capable of utilizing mannitol as a carbon source, although it only supports slow growth. Neves et al. demonstrated that the mannitol generated from glucose could be taken up rapidly after glucose had been depleted (11). In our study, we observed that a high concentration of mannitol in the supernatant appeared to inhibit mannitol production, and the mannitol adapted strains could metabolize mannitol before glucose was exhausted. For this reason we disrupted mannitol uptake. Gaspar demonstrated a complete abolishment of mannitol uptake in a lactate dehydrogenase deficient *L. lactis* strain when *mtlF* gene was deleted, which supports the notion that mannitol is solely taken up by a PEP-PTS system (13). Similarly, in this study, after *mtlF* was deleted, the cells could no longer metabolize mannitol and a more stable mannitol production was obtained. Intriguingly, we detected that lactate was formed rapidly in Ace001 after incubation for nearly 20 hours. This was unexpected as this strain lacks lactate dehydrogenase activity (20). However, in some studies, lactate formation has still been reported in the culture medium despite absence of lactate dehydrogenase activity (35), and this indicates that unknown pathways may be in operation. In the AceF strain, however, no lactate could be detected, even after 70 hours of incubation.

**Great potential for improvements**

For biosynthesis of mannitol, heterofermentative LABs have been well-studied, and good yields and titers have been reported. However, when using *L. lactis* as a production platform, it becomes possible to use different sugars for producing mannitol (9). This could lower the price of the feedstock and simplify the purification process. In this study, we successfully improved mannitol production by *L.lactis*, by using a combination of adaptive evolution and metabolic rewiring, thereby overcoming bottlenecks in metabolism and increasing the supply of NADH. The strains we have developed contain no foreign genes and are considered food-grade in many countries. The gene inactivation mutations we introduced to limit the formation

of other by-products, could in principle also be obtained via traditional mutagenesis and screening, which would make such a strain non-GMO worldwide.

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# Chapter 3. Regulation of the mannitol genes and its impact on mannitol production

This chapter is based on our work ‘‘Deciphering the regulation of the mannitol operon paves the way for efficient production of mannitol in *L. lactis*’’, which has been accepted by *Applied and Environmental and Food Microbiology*, 2021.

## 3.1 Abstract

*Lactococcus lactis* has great potential for high-yield production of mannitol, which has not yet been fully realized. In this study, we characterize how the mannitol genes in *L. lactis* are organized and regulated, and use this information to establish efficient mannitol production. Although the organization of the mannitol genes in *L. lactis* was similar to that in other Gram-positives, *mtlF* and *mtlD,* encoding the Enzyme IIA component ($EIIA^{mtl}$) of the mannitol phosphotransferase system (PTS), and the mannitol-1-phosphate dehydrogenase, respectively, were separated by a transcriptional terminator, and the mannitol genes were found to be organized in two transcriptional units: an operon comprising *mtlA*, encoding the Enzyme IIBC component ($EIIBC^{mtl}$) of the mannitol PTS, *mtlR*, encoding a transcriptional activator, and *mtlF*, and a separately expressed *mtlD*. The promoters driving expression of the two transcriptional units were somewhat similar, and both contained predicted catabolite responsive elements (*cre*). Presence of carbon catabolite repression was demonstrated, and was shown to be relieved in stationary phase cells. The transcriptional activator MtlR (*mtlR*), in some Gram-positives, is repressed by phosphorylation by $EIIA^{mtl}$, and when we knocked-out *mtlF* we indeed observed enhanced expression from the two promotors, which indicated that this mechanism was in place. Summing up, the results of our study should be useful for improving the mannitol producing capacity of this important industrial organism by using its native mechanisms.

## Importance

Mannitol has several health benefits and has been widely applied in therapeutics and foods. *L. lactis* is a promising mannitol producer due to its food-grade status, and its inherent ability to produce mannitol. However, wild type *L. lactis* strains do not generate mannitol for different reasons. To date, there have been many attempts to persuade it into producing mannitol, however, most have relied on overexpression of foreign genes, rendering these solutions GMO. In this study, we systematically characterize how the mannitol genes in *L. lactis* are regulated, and demonstrate how this impacts on mannitol production capability. We harness this information and manage to establish efficient mannitol production, without using foreign genes.



## Key words

*L. lactis*, mannitol, *mtlR*, *mtlD*, *ccpA*

## 3.2 Introduction

*Lactococcus lactis* (*L. lactis*) is a lactic acid bacterium (LAB) with a long history of safe use in the dairy and food industry. Due to its safe status, its well-known metabolism, and the many genetic tools that have been developed for it, *L. lactis* is regarded as an excellent production platform for food ingredients as well as for compounds with sensitive applications, such as therapeutics. Mannitol, a sugar alcohol, is an example of a compound that can be produced in *L. lactis*. Although the mannitol metabolism in *L. lactis* has been studied to some extent during the last two decades, still many aspects of mannitol production are unclear (1)(2).

Mannitol biosynthesis in *L. lactis*, and other homofermentative lactic acid bacteria, starts with the glycolysis intermediate fructose 6-phosphate. The mannitol 1-phosphate dehydrogenase (M1PDH) is responsible for reducing fructose 6-phosphate into mannitol 1-phosphate, which subsequently is dephosphorylated to mannitol by an uncharacterized phosphatase activity. The normal function of M1PDH is not to facilitate mannitol production, but rather growth on mannitol, and its encoding gene *mtlD* is located in an operon together with genes coding for the mannitol specific PEP (phosphoenolpyruvate)-PTS components (*mtlA* & *mtlF*) and the gene for the regulator (*mtlR*). Although *L. lactis* has the metabolic capacity for making mannitol, the relevant genes are under tight control and appear not to be expressed on glucose.

In most Gram-positive bacteria, the transcription activator MtlR controls the expression of the genes encoding M1PDH, and the D-mannitol-specific PTS components (3). MtlR from few different Gram-positive bacteria have been studied and they all share domains that have different regulatory functions depending on organism. In general, MtlR contains an N-terminal helix-turn-helix motif followed by an Mga-like domain, two PTS regulation domains (PRDs), an $EIIB^{Gat}$-like, and an $EIIA^{Mtl}$-like domain (Figure 3.1). MtlR in the lactic acid bacterium *Lactobacillus casei* is an exception, as it does not have the PRD1 and $EIIB^{Gat}$-like domains. There are several regulatory sites in MtlR that can be phosphorylated by Enzyme IIs (EIIs) and HPr, which can activate or inhibit the activity of MtlR. In the presence of an efficiently metabolized PTS substrate, e.g. glucose, HPr is barely phosphorylated (4), and this prevents activation of MtlR. Another regulatory mechanism, which turns off MtlR when mannitol is absent, is the mannitol specific PTS component EIIA, which can phosphorylate the $EIIB^{Gat}$-like domain of MtlR, leading to inactivation of MtlR (5). In addition to these forms of regulation the classical carbon catabolite repression is also involved in the regulation of the mannitol operon (6) and together these three regulatory mechanisms probably explain why

*mtlD* expression normally is repressed during growth on other sugars, and why mannitol is not produced.

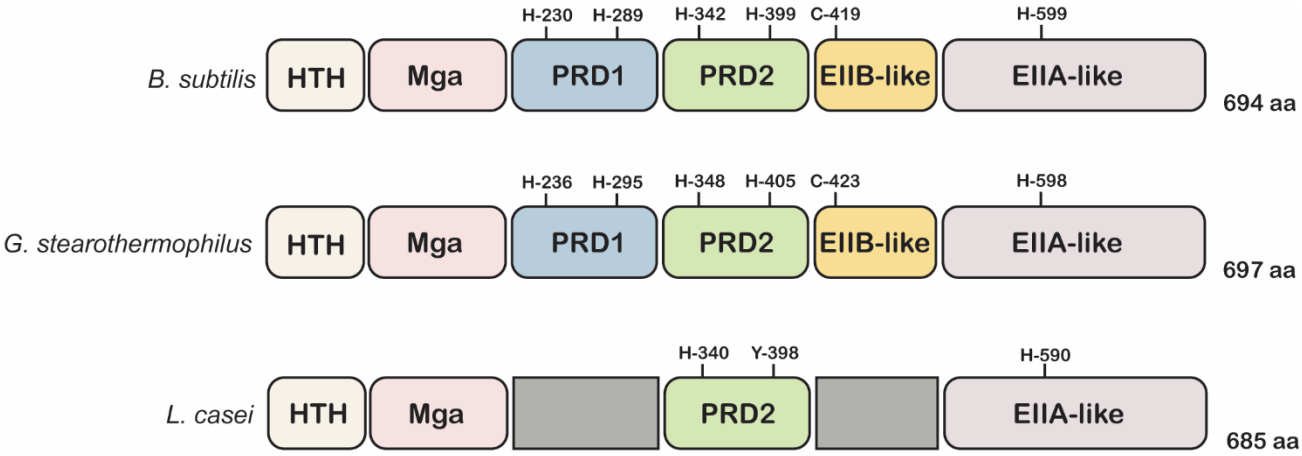


**Figure 3.1. Protein structure of the characterized mtlR from *B. subtilis, G. stearothermophilus and L. casei.*** The phosphorylation sites were indicated with the amino acid and their position.

There have been many efforts to achieve mannitol production in *L. lactis*. Gaspar et al. (7), reported that an *L. lactis* strain deficient in lactate dehydrogenase (*ldh*) formed small amounts of mannitol (0.7 mM) when growing on glucose, however, after glucose depletion mannitol was metabolized. The same researchers also found that non-growing cells resuspended in buffer, were able to convert 33% of the glucose metabolized into mannitol. Wisselink et al., 2005 demonstrated that the M1PDH had an impact on mannitol production, but the effect was only seen clearly in a lactate dehydrogenase deficient strain, even under non-growing conditions. By heterologous overexpression of the mannitol-1-phosphatase from *Eimeria tenella* and a M1PDH from *Lactobacillus plantarum* in a LDH deficient strain, efficient mannitol production (9.0 g/L, 50% yield) was accomplished in growing *L. lactis* cells for the first time (8). All reported cases where mannitol is produced using growing cells, involve introduction of foreign genes in *L. lactis* in combination with additional modifications to metabolism. Since such strains are genetically engineered, their use in food fermentations is precluded. To address this problem we recently attempted a different strategy, namely adaptive evolution on mannitol, where the aim was to debottleneck mannitol catabolism, in the hope that this could result in more efficient production of mannitol from glucose. It was indeed possible to enhance growth on mannitol, and the mutants obtained contained a mutation upstream *mtlA*, which greatly enhanced expression of the mannitol operon. By further

eliminating competing NADH consuming pathways, it was possible to achieve mannitol production from glucose with high yield (60%) (Chapter 1).

In this study, we further characterize the effects of those mutations and study the roles that MtlR and CcpA have in regulating mannitol production from glucose. Based on the knowledge accumulated we manage to establish efficient mannitol production.

**Table 3.1. Strains and plasmids used in this work**

| Strain and plasmid | Relevant genotype | Source or reference |
|---|---|---|
| **Strains** | | |
| **MG1363** | Wild-type *L. lactis* subsp. *cremoris* | (9) |
| **MG363-M** | MG1363 adapted on mannitol | Chapter 2 |
| **Ace001** | MG1363 *Δ*$^{3}$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA* | (10) |
| **Ace001-M** | Ace001 adapted on mannitol | Chapter 2 |
| **AceF** | Ace001-M *ΔmtlF* | Chapter 2 |
| **AceR** | Ace001-M *ΔmtlR* | This work |
| **Ace001C** | Ace001 *ΔccpA* | This work |
| **AceCF** | Ace001-M *ΔmtlF, ΔccpA* | This work |
| **AceCR** | Ace001-M *ΔmtlR, ΔccpA* | This work |
| **Ace001-mtlR** | *mtlR* overexpressed Ace001, 20%TPI promoter | This work |
| **MC1000** | *E.coli* cloning host | (11) |
| **DH10B** | high-efficiency competent *E. coli* host | |
| **Plasmids** | | |
| **pTD6** | pTD6 A derivative of pAK80 containing a *gusA* reporter gene | (12) |
| **pPmtlA$^{WT}$** | pTD6 with wild type *mtlA* promoter preceding *gusA* reporter gene | This work |

| | | |
|---|---|---|
| **pPmtlA$^{mut}$** | pTD6 with C-39T mutant *mtlA* promoter preceding *gusA* reporter gene | This work |
| **pPmtlA$^{mutII}$** | pTD6 with G-46T mutant *mtlA* promoter preceding *gusA* reporter gene | This work |
| **pPmtlD** | pTD6 with *mtlD* promoter preceding *gusA* reporter gene | This work |
| **pPmtlAD** | pTD6 with fused *mtlA* and *mtlD* promoter preceding *gusA* reporter gene | |
| **pJET1.2** | high-efficiency cloning plasmid for blunt-ended ligation | ThermoFisher Scientific |
| **pG$^{+}$host8** | *E. coli*/*L. lactis* shuttle vector, Tet$^{R}$, thermosensitive replicon | (13) |
| **pKmtlR** | Used for knocking out *mtlR* | This work |
| **pKCcpA** | Used for knocking out *ccpA* | This work |

## 3.3 Methods

### Bacterial Strains and Growth Conditions

*Lactococcus lactis* subsp. *cremoris* MG1363 and derivatives were used in this study (described in Table 3.1). *L. lactis* strains were cultured in M17 broth supplemented with 1% glucose (Sigma Aldrich) at 30°C, with shaking at 240 rpm. When needed 5 μg/ml tetracycline was used for selection, and 2 μg/ml in physiological growth experiments. The optical density of cell suspensions was measured using a spectrophotometer (Shimadzu) at 600 nm. Resting cells preparation and two-step fermentation were performed as described previously (14).

### Methods and tools for bioinformatics analysis

The information about mannitol genes in different bacteria was obtained from NCBI: NC_009004 (*L. lactis*), NC_000964 (*Bacillus subtilis*), U18943 (*Geobacillus stearothermophilus*), U53868 (*Clostridium acetobutylicum*) and AE014133 (*Streptococcus mutans*). The bioinformatics analysis was performed using different webtools: BDGP(15) and

SAPPHIRE https://sapphire.biw.kuleuven.be/ for promoter prediction; De Novo DNA (https://www.denovodna.com) for predicting ribosome binding site; Arnold (http://rssf.i2bc.paris-saclay.fr/toolbox/arnold) for predicting transcriptional terminators.

**Molecular techniques**

Electrocompetent cells were prepared using the protocol of Holo & Nes (16), using GM17 medium containing either 0.5% (for strains AceCF & AceCR) or 2% glycine and 0.25 M sucrose. The medium was filtered. Electroporation was performed as as previously described by Holo and Nes using MicroPulser Electroporator (Bio-Rad) (16). Phusion DNA Polymerase (Thermofisher Scientific) was used for PCR amplifications. Zyppy plasmid miniprep kit (Zymo Research) was used for extracting the plasmids from *L. lactis* after a pre-treatment with 20mg/mL lysozyme (Fluka, 7,000U/mg) at 37°C for 2 hours. FastDigest restriction enzymes SalI, SacI and BamHI were obtained from Thermofisher Scientific.

- Deletion of *mtlR* in strain Ace001M

To delete *mtlR* in the strain Ace001M, $pG^+$host8, a plasmid with a thermosensitive replicon was used. Two synthetic DNA fragments, representing the upstream and downstream regions of *mtlR*, and able to overlap, were ordered from Integrated DNA Technologies: a 985 bp fragment upstream of *mtlR* into which a SalI restriction site had been introduced in the *mtlR* distal end, and a 993 bp fragment downstream of *mtlR*, with a BamHI restriction site added in the *mtlR* distal region. After fusing the two fragments they were inserted into pJET1.2 using CloneJET PCR Cloning Kit by blunt ligation as described in the manual and transformed into *E. coli* DH10B. The insert was excised as a SalI/BamHI fragment and introduced into the corresponding sites in $pG^+$host8 using *E. coli* MC1000 as host. The resulting plasmid, designated pKmtlR ($pG^+$host8/$mtlR_{up}$-$mtlR_{dwn}$), was subsequently used to delete *mtlR* in Ace001-M in the same manner as described previously (14). The Ace001-M derivative with deleted *mtlR* was designated AceR.

- Construction of strains with deleted *ccpA*

The same approach used to delete *mtlR*, was taken for deleting *ccpA*. In this case, a 978 bp fragment upstream of *ccpA* equipped with a SalI restriction site, and a 984 bp fragment downstream of *ccpA* with a BamHI site were used. The subsequent cloning steps were identical to the ones mentioned above. For obtaining the strains with deleted *ccpA*, we relied on the slow growth phenotype of *ccpA* mutants (17), and for this reasons small colonies were selected and

investigated. Deletion of *ccpA* was done for strains AceF, Ace001 and AceR, and the derivatives were designated AceCF, Ace001C and AceCR respectively.

- Overexpression of *mtlD* and *mtlR*

An expression cassette consisting of a promoter, a ribosome binding sequence and *mtlD* was generated using a nested PCR approach. First, a fragment containing the entire *mtlD* gene was amplified using primers 5'-ATTTCGGGAGACACATCTGGC-3' and 5'-GTGTTCTCGCTTCGCATCAG-3'. For the second PCR a forward primer containing the 20% TPI promoter sequence(18) and a ribosome binding sequence was used 5'-CACGCGTCGACATAGATTAGTTTATTCTTGACACTACAAGCTAAATGTGGTATAA TCCCATAGATATACTAGGTAAGTAATAAAATATTCGGAGGAATTTTGAAATGAA AAAAGCAGTACATTTTGGTGCAGGAAAT -3' together with the following reverse primer 5'- ATCCGGAGCTCATATTCTCTGTCTACTTGCTGTCAT -3'. The same approach was used for generating an expression cassette for *mtlR*: first, a fragment containing the entire *mtlR* gene was amplified using primers 5'- AAGAAGAATTCACGGCAATA -3' and 5'-ATCGCTGAAACATAATTTGAG -3'. For the second PCR the following primers were used: 5'- CACGCGTCGACATAGATTAGTTTATTCTTGACACTACAAGCTAAATGTGGTA-TAATCCCATAGATATACTAGGTAAGTAATAAAATATTCGGAGGAATTTTGAAATG TTTTTAACAAGTCGTGAG -3' & 5'- ATCCGCTCGAGCTAATCACCATACTGTTT-AACAGC -3'. All primers are ordered from IDT company. The amplified fragments were digested with restriction enzymes and inserted into pTD6 and the constructs introduced into Ace001 and verified by Sanger sequencing (Macrogen).

**Quantifying mannitol production by High-Performance Liquid Chromatography (HPLC)**

For quantifying glucose and mannitol, we used an HPLC equipped with a BioRad Aminex HPX-87H column (BioRad) and a RI-101 detector (Shodex). The mobile phase consisted of 5 mM $H_2SO_4$ at a flow rate of 0.5 ml/min. The column oven temperature was set to 60°C. The samples for HPLC analysis were filtered using 0.22 μM filters (Labsolute) and loaded to HPLC immediately after sampling at appropriate cells density.

**M1Pase activity assay**

For detecting M1Pase activity, exponentially growing culture samples were quenched in wet ice. The cells were washed with ice-cold MES buffer (50 mM MES, 10 mM $MgCl_2$, pH=7.0), and resuspended to a density of $OD_{600}$=50.0. For permeabilizing the cells, 12.5 μl 0.1% SDS

and 25 µl $CHCl_3$ was added. After vigorously votexing for 10 s and equilibrium at 30°C for 5 min, an appropriate volume of M1P (Sigma Aldrich ) was added to a final concentration of 5 mM to initiate the reaction, meanwile, for negative controls, the same volume of MES buffer was added. After 1 h incubation at 30°C, cells were centrifuged and the supernatant was filtered with 0.22 µM filters (Labsolute). Finally, the filtered supernatant was immediately analyzed by HPLC to determine formation of mannitol. M1Pase activity was descried as the amount of mannitol (mM) formed per hour.

**β-glucuronidase assays**

Strains were cultured in M17 broth supplemented with 1% of the indicated sugar and 2 µg/ml tetracycline. Cells were harvested after reaching $OD_{600}$=1.0 or in the stationary phase ($OD_{600}$=4.0), and quenched on wet ice followed by centrifugation at 4°C. Harvested cells were washed in ice cold PBS and resuspended in ice-cold Z buffer (0.06 M $Na_2HPO_4$, 0.04 M $NaH_2PO_4$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05M β-mercaptoethanol, pH=7). Cells were appropriately diluted and the cell density was measured at $OD_{600}$. For permeabilization, 12.5 µl 0.1% SDS and 25 µl $CHCl_3$ were added, and the suspension vortexed vigorously. After equilibrium at 30°C for 5 min, 100 µl of 4 mg/ml PNPG was added, and the time needed for developing a yellow color recorded. To stop the reaction 800 µl $Na_2CO_3$ was added. Absorbance at 420 nm and 550 nm were measured. Promoter strength was calculated as $1000*(OD_{420}-1.75*OD_{550})/(time*OD_{600}*ml\ sample)$ and describe as Miller units.

**RNA sequencing**

The cells of Ace001M grown in mannitol or glucose were harvested at exponential or stationary phase, besides, AceCM and AceCR grown in glucose were harvested at exponential phase. Total RNA of all these cells were extracted by RNeasy Kit (Qiagen) and purified by using RNase-Free DNase Set (Qiagen) according to the manuals. All treatments were duplicated. All RNA samples were sent to BGI (Hong Kong) and stranded-specific & rRNA-depleted library preparation were performed. Then PE100 sequencing were performed on DNBSWQ platform. 2Gb clean data per sample were generated. Trimmed reads were obtained from BGI. All data were then import to and analyzed by Geneious Prime V11.0.4. MG1363 genome NC_009004 were used as reference sequence for genome alignment, by using the default settings in Geneious Prime. Expression level (RPKM, FPKM and TPM) of genes in each treatment was performed by the method Count as Partial Matches based on annotated CDS region. DESeq2 was used for comparing the expression level among different treatments.

## 3.4 Results

### 3.4.1 Organization of mannitol genes in *L. lactis* and effect of mutations arising from adaptive evolution on mannitol

#### Organization of the mannitol genes in *L.lactis*

As mentioned above, we recently reported that adaptive laboratory evolution combined with systematic elimination of NADH consuming reactions can lead to efficient mannitol production, by *L. lactis*, from glucose. The strains obtained after adaptive evolution contained two different mutations, C-39T and G-46T, both in the *mtlA* promoter region, which we found were critical for mannitol production.

To better understand how these mutations affected mannitol production, we first examined the genetic arrangement of the mannitol genes in *L. lactis* and compared our findings to those previously reported for other Gram-positive bacteria. As shown in Figure 3.2(a), the mannitol genes appear to be organized in a similar manner in all the bacteria investigated, except for *B. subtilis*, where the gene encoding the regulator MtlR is not part of the operon. Nevertheless, we observed two distinct features for the *L. lactis* genes. The first one was that the spaces beween *mtlR* & *mtlF*, and between *mtlF* & *mtlD* appear longer in *L. lactis*, than for the other organisms. Another difference is that there is a predicted rho-independent terminator between *mtlF* and *mtlD*, suggesting that *mtlD* is transcribed from its own promoter. Interestingly, the gene just after *mtlD*, a characterized 6S RNA (16) may also be affected by the promoters upstream of *mtlD*, since no transcriptional terminator can be identified between the 6S RNA gene and *mtlD*.

The promoter regions upstream *mtlA* and *mtlD*, in *L. lactis*, were also analyzed, and were found to share more than 47% identity. Both regions have putative MtlR binding sequences and *cre* sites that are well-conserved. Furthermore, as shown in Figure 3.2(b), the two mutations, C-39T and G-46T were both found to locate in the predicted putative *cre* site, where C-39T also overlapped with the putative -10 element region, suggesting that the mutations affect the expression of mannitol operon.

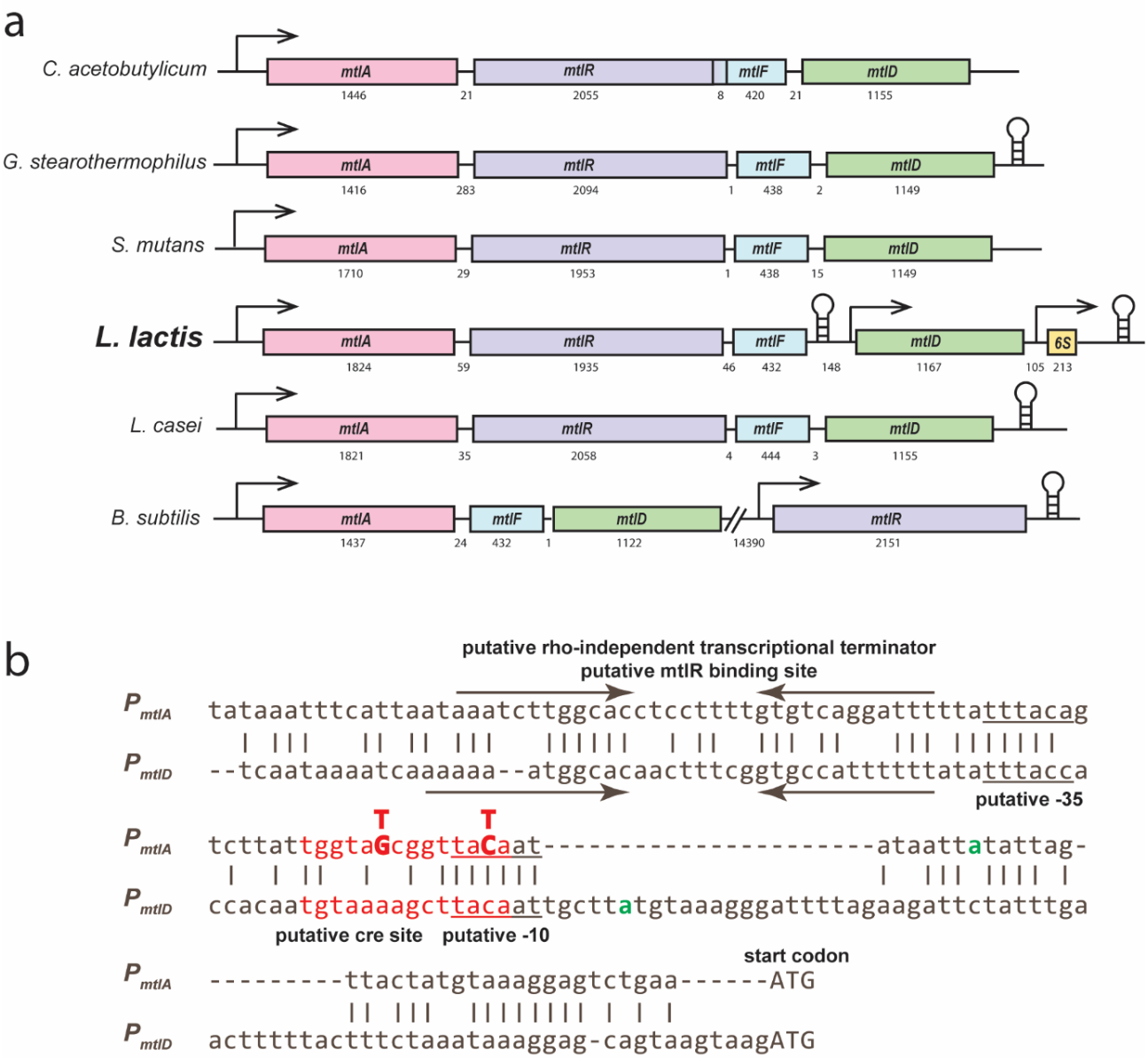


**Figure 3.2. Arrangement of the mannitol genes in *L. lactis* and other selected firmicutes, as well as putative promoters upstream *mtlA* & *mtlD* in *L. lactis*.** In a), arrows represent characterized or predicted promoters. The numbers beneath the genes indicate either gene length or length of the spacers between indicated genes. 6S, 6S RNA. In b), putative transcriptional elements are indicated. The transcriptional start sites are indicated with green color; *cre* sites are indicated with red color, the inverse repeat sequence, i.e., the *mtlR* binding site was indicated with arrows; -10 and -35 elements are underscored. The mutations C-39T and G-46T (the numbers indicate the distance from start codon ATG) in *mtlA* promoter region gained during mannitol adaptation are indicated with a red capital bold letter.

**Adaptive laboratory evolution on mannitol lead to mutations in the *mtlA* promoter which enhance expression**

To assess the effect of these mutations on expression from the *mtlA* promoter, transcriptional fusions between the *mtlA* promoter and the reporter gene *gusA* (encoding β-glucuronidase) were constructed based on the reporter vector pTD6. As shown in Table 3.2, on glucose, a low promoter activity, no more than 0.1 MU (Miller Units), was observed for the wild-type promoter. In contrast, the promoters containing the C-39T or G-46T mutations were significantly stronger, in particular the C-39T mutation, which was 40 fold stronger than the promoter containing the G-46T mutation. The β-glucuronidase activity measured, depended on the host, e.g. the activity of the C-39T promoter in the mannitol adapted strain MG1363M, having the C-39T mutation in its genome, was 52.16 MU, whereas the activity in the wild-type strain MG1363 only reached 15.6 MU. As MtlR is expressed together with *mtlA* and the mannitol PTS genes (one transcriptional unit), the C-39T mutation in MG1363M would result in higher expression of MtlR, which could in turn explain the higher activity of the *mtlA* promoter in the MG1363M background. On mannitol, as expected, we found a higher activity, and whereas the wild-type promoter reached 449 MU, the C-39T promoter activity resulted in more than 4000 MU.

**Table 3.2. Effect of mutations in the mannitol operon promoter on expression level**

| Sugars | Host | $P_{mtlA,WT}$ | $P_{mtlA,C\text{-}39T}$ | $P_{mtlA,G\text{-}46T}$ |
|---|---|---|---|---|
| Glucose | MG1363 | 0.01±0.01 | 15.6±0.9 | 0.43±0.01 |
| Glucose | MG1363M | 0.06±0.01 | 52.16±2.0 | 1.17±0.11 |
| Mannitol | MG1363M | 449±5 | 4415±56 | 4183±34 |

$P_{mtlA,WT}$, wild type *mtlA* promoter; $P_{mtlA,C\text{-}39T}$, *mtlA* promoter containing the C-39T mutation; $P_{mtlA,G\text{-}46T}$, *mtlA* promoter containing the G-46T mutation. The activities were measured using cells in the exponential phase, and shown in Miller Units. The experiments were carried out in duplicates, and standard deviations are indicated.

Based on this information, the promoter mutations in the adapted strains appear to enhance the expression of *mtlA*, *mtlR* and *mtlF* and possibly *mtlD*. We previously found that deleting *mtlF* in *L. lactis* had a beneficial effect on mannitol production (14), while others have found that strains lacking either *mtlA* or *mtlF* perform similarly in terms of mannitol production (7).

### 3.4.2 Characterization of the regulatory mechanism for *L. lactis* mannitol operon

#### The promoters upstream *mtlA* and *mtlD* ($P_{mtlA}$ & $P_{mtlD}$) are transcribed separately and are both activated by MtlR

To investigate the promoter upstream *mtlD*, we inserted it into the promoter probe vector pTD6, and introduced the plasmid into Ace001 (MG1363 *Δ*$^3$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA*), its mannitol adapted version (Ace001M), and Ace001M lacking MtlR (AceR). Ace001M carries the C-39T mutation in *mtlA* promoter. We also introduced the previously constructed *mtlA* promoter-*gusA* fusion in the same strains. As shown in Figure 3.3, on glucose, in the AceR background, both promoters were less active than in the parental strain Ace001M. In Ace001M, due to the C-39T mutation, the *mtlA*/*mtlR*/*mtlF* transcriptional unit is expressed to a higher level, leading to more of the activator MtlR. These findings clearly demonstrated that the promoter upstream *mtlD* was subjected to regulation by MtlR, which was expected due to the presence of a predicted MtlR binding site in the *mtlD* promoter. Interestingly, the promoter activities were much higher for cells in stationary phase than in exponential phase and for both promoters, a large stationary phase induction was observed on mannitol.

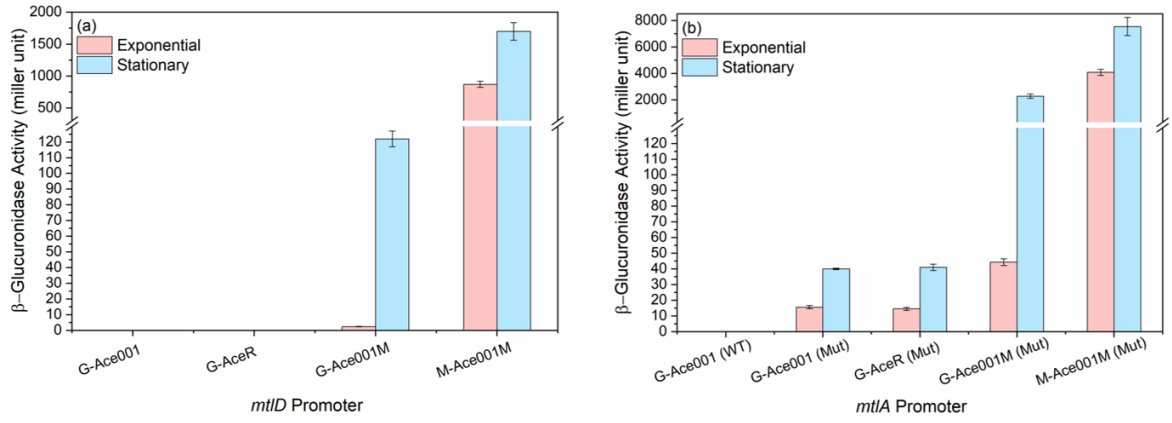


**Figure 3.3. Expression of the *mtlD* promoter, and of wild-type & mutated *mtlA* promoters in different strain backgrounds on glucose and mannitol.** (a) *mtlD* promoter expression. (b) *mtlA* promoter expression. WT, wild type *mtlA* promoter; Mut, *mtlA* promoter with C-39T mutation. The carbon source is indicated with G (1% glucose) or M (1% mannitol). The hosts are either Ace001 (MG1363 *Δ*$^3$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA*), Ace001M (Ace001 adapted on mannitol), or AceR (Ace001M *ΔmtlR*). Cells harvested in exponential phase are indicated with red color, while cells harvested in stationary phase are indicated with blue color. Experiments were carried out in duplicates, and standard deviations are indicated by error bars.

To investigate if the expression of *mtlD* could be affected by the promoter upstream *mtlA* ($P_{mtlA}$), we fused the mutated *mtlA* promoter (C-39T) with the *mtlD* promoter, including the transcriptional terminator upstream *mtlD*, and introduced the construct into the reporter vector pTD6. In the Ace001M background, a low expression level was detected, only 1.82±0.13 MU, which is similar to that found for *mtlD* promoter alone. This demonstrates that *mtlD* is not part of the *mtlA-mtlR-mtlF* operon.

**Deleting *ccpA* increases expression of the mannitol genes**

The mannitol genes are subjected to carbon catabolite repression, and this might have a negative effect on mannitol production from glucose. To investigate this, we deleted *ccpA* in Ace001, thereby generating Ace001C. As shown in Figure 3.4, by deleting *ccpA*, the *mtlA* and the *mtlD* promoters were both derepressed, and activities of 588 MU and 405 MU, respectively were measured in the exponential phase (Table 3.3). Furthermore, we found that the wild type *mtlA* promoter and *mtlA* promoter from the mannitol-adapted strains had similar activities in the *ΔccpA* background, demonstrating that the mutation had affected the functionality of the *cre* site (Figure 3.4). Interestingly, the promoter activities were two to three fold higher in the stationary phase, even in the presence of CcpA.

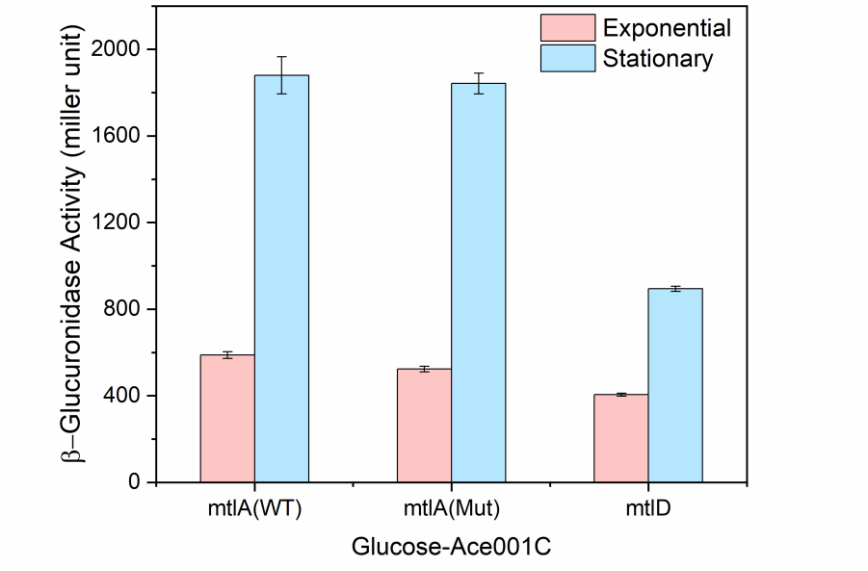


**Figure 3.4. The activity of *mtlA* and *mtlD* promoters in Ace001C, a strain lacking CcpA.** WT, wild type *mtlA* promoter; Mut, *mtlA* promoter with C-39T mutation. Cells are grown in 1% glucose and harvested at exponential phase (red color), stationary phase (blue color). Experiments were carried out in duplicates and standard deviations are indicated by error bars.

**MtlR is needed for high-level expression of the mannitol genes, and its level can be enhanced by deleting *mtlF***

For investigating the extent to which MtlR affects expression of mannitol genes, we deleted *ccpA* in AceR, which lacks *mtlR*, thus generating AceCR. As shown in Figure 3.5, compared with AceR (Figure 3.3), deleting *ccpA* (AceCR) only had a small positive effect on the *mtlA* promoter, and the *mtlD* promoter had no activity. These results clearly demonstrate that the mannitol genes in *L. lactis* are tightly regulated by MtlR. To investigate whether eliminating CcpA could be beneficial for mannitol production, we deleted *ccpA* in strain AceF, which harbors the C-39T promoter mutation and in which *mtlF* has been deleted; the outcome was strain AceCF. As observed earlier, without *mtlF*, the activity of *mtlA* and *mtlD* promoters were dramatically increased to 3787 MU and 1955 MU, respectively, in the exponential phase, and increased to 6055 MU and 3664 MU in the stationary phase (Figure 3.5 and Table 3.3).

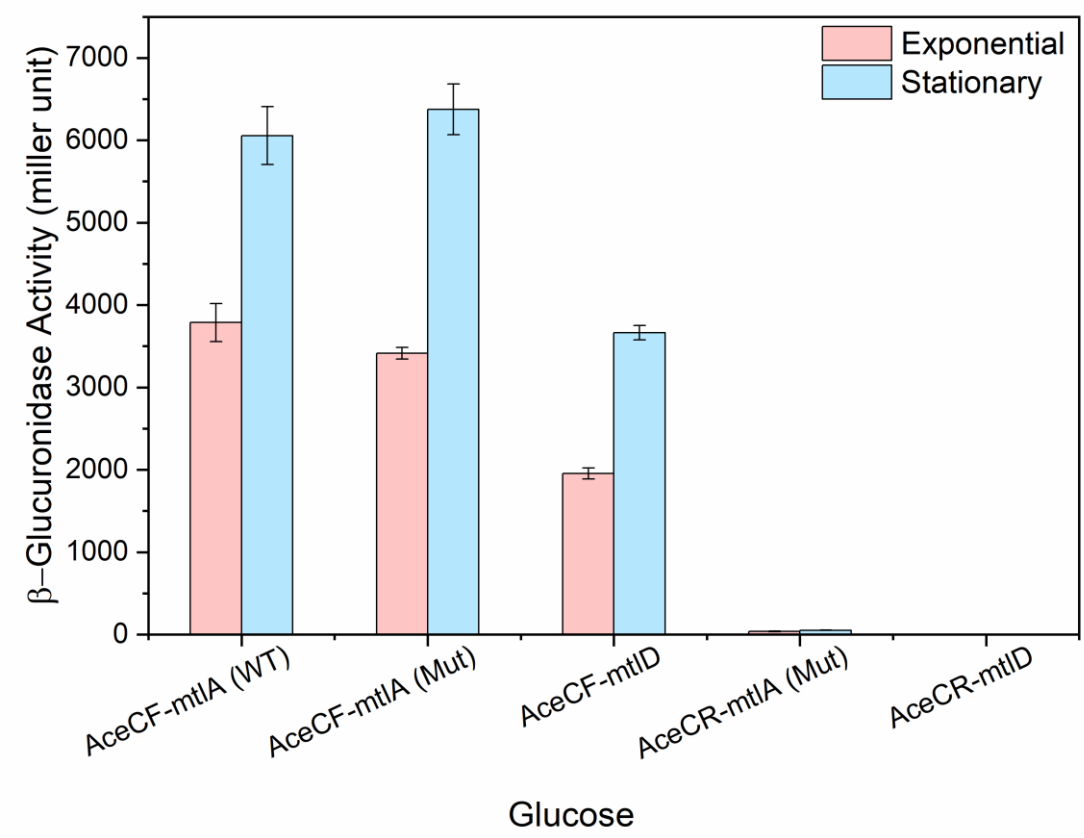


**Figure 3.5. The activity of *mtlA* and *mtlD* promoters in AceCF and AceCR.** WT, wild type *mtlA* promoter; Mut, *mtlA* promoter with C-39T mutation. Cells were growing in 1% glucose and harvested at exponential phase (red color), stationary phase (blue color). Experiments were carried out in duplicates and standard deviations are indicated with error bars.

**Expression of mannitol operon in different sugars**

We have shown mannitol production in *L. lactis* is strongly correlated with the expression level of mannitol operon, which is subjected to CCR. To test how the expression level of mannitol operon changed when cells incubated with different sugars, which might alleviate CCR, GusA activity assay was performed. In contrast to the promoter activity obtained from Ace001M growing in glucose, which giving 44.3 MU of $P_{mtlA}^{mut}$, and 2.38 MU of $P_{mtlD}$, as shown in Table 3.3, Ace001M growing in fructose, galactose and maltose resulted in higher promoter activity both in $P_{mtlA}^{mut}$ and $P_{mtlD}$. In addition, cells grew in maltose giving the highest expression of mannitol operon, which is higher than at least 4 fold than that in glucose.

**Table 3.3. Influence of strain background, carbon source and growth phase on the activity of *mtlA* and *mtlD* promoters**

| Sugar | Strains | Promoters | Miller units | |
|---|---|---|---|---|
| | | | Exponential | Stationary |
| Glucose | Ace001 | *mtlD* | 0.00±0.00 | 0.00±0.00 |
| Glucose | AceR | *mtlD* | 0.01±0.01 | 0.01±0.00 |
| Glucose | Ace001M | *mtlD* | 2.38±0.10 | 122±5 |
| Mannitol | Ace001M | *mtlD* | 871±46 | 1699±137 |
| Glucose | Ace001 | $mtlA^{WT}$ | 0.03±0.00 | 0.05±0.01 |
| Glucose | Ace001 | $mtlA^{Mut}$ | 15.6±1.1 | 40.0±0.4 |
| Glucose | AceR | $mtlA^{Mut}$ | 14.5±0.9 | 41±2 |
| Glucose | Ace001M | $mtlA^{Mut}$ | 44.3±2.2 | 2273±164 |
| Mannitol | Ace001M | $mtlA^{Mut}$ | 4078±230 | 7539±687 |
| Glucose | Ace001C | $mtlA^{WT}$ | 588±15.7 | 1880±86 |
| Glucose | Ace001C | $mtlA^{Mut}$ | 523±13.1 | 1842±48 |
| Glucose | Ace001C | *mtlD* | 405±6.6 | 894±12 |
| Glucose | AceCF | $mtlA^{WT}$ | 3787±232 | 6055±351 |
| Glucose | AceCF | $mtlA^{Mut}$ | 3413±70 | 6375±308 |
| Glucose | AceCF | *mtlD* | 1955±65 | 3664±88 |
| Glucose | AceCR | $mtlA^{Mut}$ | 39.0±3.5 | 52.2±1.5 |
| Glucose | AceCR | *mtlD* | 0.00±0.00 | 0.06±0.01 |
| Fructose | Ace001M | $mtlA^{Mut}$ | 102±3.47 | - |
| Fructose | Ace001M | *mtlD* | 3.64±0.17 | - |
| Galactose | Ace001M | $mtlA^{Mut}$ | 130±5.01 | - |
| Galactose | Ace001M | *mtlD* | 14.2±0.59 | - |
| Maltose | Ace001M | $mtlA^{Mut}$ | 218±5.45 | - |
| Maltose | Ace001M | *mtlD* | 61.9±2.32 | - |

Note: The activity of *mtlA* and *mtlD* promoters in stationary Ace001M incubated with fructose, galactose and maltose have not been characterized.

**Inactivating CcpA enables mannitol production in non-mannitol adapted strains**

To quantify the effect that MtlR has on the expression level of the mannitol genes, on mannitol catabolism and on mannitol production from glucose, we overexpressed *mtlR* from a promoter of intermediate strength in the non-mannitol producer Ace001 (MG1363 *Δldh ΔldhB ΔldhX Δpta ΔadhE ΔbutBA*). As shown in Table 3.4, this enabled mannitol production from glucose. Deleting *mtlR* in the mannitol producer Ace001M, abolished both growth on mannitol and mannitol production from glucose, which indicates that MtlR has a key role in both mannitol catabolism and production. AceCF and AceF were both able to produce mannitol, but unable to grow on mannitol, where the latter was expected since *mtlF* encodes a critical component of the mannitol PTS.

**Table 3.4. Effect of overexpressing/deleting *mtlR* in different *L. lactis* backgrounds**

| Strain | Genotype | Growth on mannitol | Mannitol production |
|---|---|---|---|
| Ace001 | MG1363 *Δldh*, *ΔadhE*, *Δpta*, *ΔbutBA* | - | - |
| Ace001M | Ace001 adapted on mannitol | + | + |
| AceF | Ace001M *ΔmtlF* | - | + |
| Ace001-mtlR$^{\uparrow}$ | *mtlR* overexpressed Ace001 | - | + |
| AceR | Ace001M *ΔmtlR* | - | - |
| Ace001C | Ace001 *ΔccpA* | + | + |
| AceCF | AceF *ΔccpA* | - | + |
| AceCR | AceR *ΔccpA* | - | - |

For assessing growth, we used the following criterium: Cell density attained in M17 containing mannitol should be above $OD_{600}$ > 0.9 after 24 hours of incubation (M17 without added sugar/mannitol supports growth to approximately $OD_{600}$ around 0.8.). For assessing mannitol production, if more than 0.001 g/L mannitol is formed, we interpret this as an ability to produce mannitol from glucose.

**MtlR has no influence on the mannitol-1-phosphatase activity**

In *L. lactis*, two activities are needed for mannitol production to occur (Figure 3.6); the first is mannitol-1-phosphate dehydrogenase (*mtlD*), and the second is a phosphatase activity that can convert mannitol-1-phosphate into mannitol, where the latter has not been unambiguously identified. The impact that MtlR has on the expression of the mannitol genes is clear, however,

is it possible that MtlR could serve as an M1Pase or be able to increase the M1Pase activity in cells, as MtlR contains several phosphoryl transfer domains. For testing this, we measured the M1pase activity in Ace001-mtlR and AceR. We finally detected similar M1Pase activity in Ace001-mtlR AceR and Ace001 (0.48, 0.49 and 0.49 respectively). Based on these results, we concluded that MtlR is not responsible for the M1Pase activity in *L. lactis*.

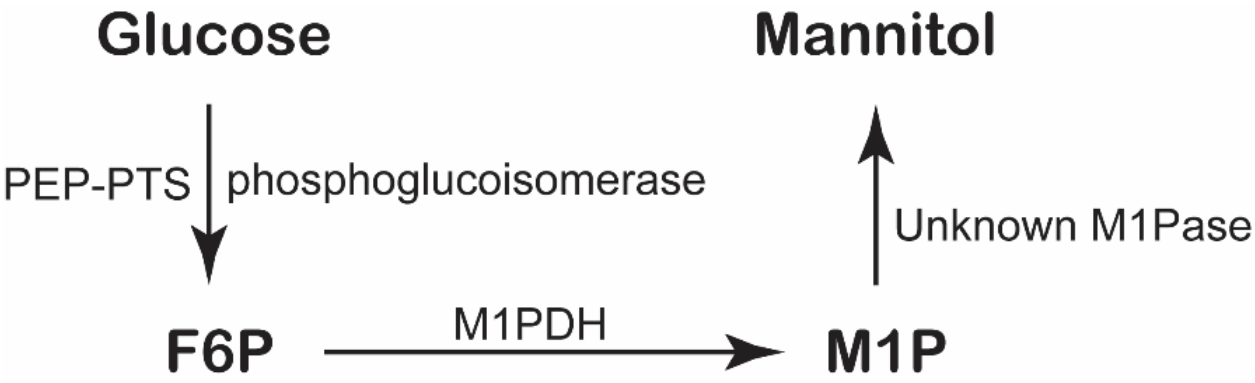


**Figure 3.6. Scheme of mannitol production in *L. lactis*.** PEP-PTS, phosphoenolpyruvate (PEP)-dependent glucose phosphotransferase system (PTS); F6P, fructose-6-phosphate; M1PDH, mannitol-1-phosphate 5-dehydrogenase; M1P, Mannitol-1-phosphate

### 3.4.3 RNA sequencing as a means to validate regulation

Transcriptional fusion analysis is an excellent approach to investigate regulation of gene expression, however, we decided to use RNA sequencing as a second layer of validation. As shown in Table 3.5, RNA sequencing clearly indicated that expression of the mannitol genes was highly induced for cells grown on mannitol, and the expression of *mtlA*, *mtlR*, *mtlF*, and *mtlD* was increased by 12, 14, 14, and 26 times, respectively, and decreased up to two times in stationary phase. In contrast, for cells grown on glucose, the expression of the genes increased by a factor of 1.8 to 4.4 at stationary phase. Deletion of *ccpA* and *mtlF* resulted in 11, 24 and 63 times increased expression of *mtlA*, *mtlR* and *mtlD* genes, respectively. In contrast, by deleting *mtlR*, a dramatic decrease in expression level by a factor of 161 and 871 times was observed for *mtlA* and *mtlD* genes, respectively.

In *L. lactis*, a 6S RNA gene is located downstream of the *mtlD* gene. Since no transcriptional terminator precedes the *6S RNA*, the expression of this gene is deduced to be affected by the

expression of mannitol operon. From Table 3.5, a 3-times upregulation of this gene was observed for cells growing in mannitol. For mannitol-grown cells in stationary phase, the expression level of the 6S RNA gene was slightly increased by around 1.47 fold. Furthermore, by deleting *ccpA*, the expression level of this gene increased by 5.9 times. By further deleting *mtlR*, the expression level decreased 3.7 times, strongly indicating that the expression of this *6S RNA* indeed is affected by the expression level of the mannitol operon. Furthermore, for AceCR, where the expression level of mannitol operon is extremely low, deletion of *ccpA* resulted in a higher expression level of *6S RNA* compared with that in Ace001M growing in glucose at exponential phase, suggesting this gene is also regulated by *ccpA*.

*ccpA* is a global transcriptional regulator affected the expression of a large number of genes in *L. lactis*. To have a deeper understand of in what way it affects the expression of mannitol operon, we also analyzed *ccpA* expression from our sequencing data. In Table 3.5, the expression level of *ccpA* did not change too much between exponential phase and stationary phase. It had a higher expression level in stationary phase and seemed to be slightly induced by mannitol in exponential phase.

**Table 3.5. Transcriptional expression level of genes in mannitol operon, *6S RNA* and *ccpA* in different genetic background, growing phase and carbon source**

| Gene | AceCR | Ace001M, G | | Ace001M, M | | AceCF |
|---|---|---|---|---|---|---|
| | Exp. | Exp. | Sta. | Exp. | Sta. | Exp. |
| *mtlD* | -5.93 | -2.15 | 0 | 2.55 | 2.57 | 3.84 |
| *mtlF* | -3.30 | -1.25 | 0 | 2.58 | 2.25 | ND |
| *mtlA* | -5.00 | -1.09 | 0 | 2.49 | 1.64 | 2.32 |
| *mtlR* | ND | -0.81 | 0 | 3.02 | 2.06 | 3.80 |
| *6S RNA* | -0.97 | -1.63 | 0 | 0 | 0.56 | 0.92 |
| *ccpA* | ND | 0.05 | 0 | 0.65 | -0.16 | ND |

Differential expression analysis for *mtlD*, *mtlF*, *mtlA*, *mtlR*, 6S RNA and *ccpA*, in different conditions, as determined by log2 fold change, and using glucose grown Ace001M cells in stationary phase as reference. ND, not detectable; Exp., stands for exponential phase; Sta., stands for stationary phase; Cells growing in glucose or mannitol were indicated with G or M respectively;

## 3.5 Discussion

In *L. lactis*, *mtlD* encodes M1PDH, which is a key enzyme in mannitol metabolism. In this study, we found that MtlR is essential for maintaining the expression of *mtlD*, thus allowing for mannitol production in non-growing or slowly growing cells.

### The phosphatase responsible for dephosphorylating mannitol-1-phosphate remains to be found

Although several studies have shown that *L. lactis* is able to convert M1P into mannitol, the responsible enzyme has not been identified (7)(26). It has been suggested that a mannitol specific PTS component could be involved in this, e.g. the $EIIBC^{mtl}$, as it is able to transfer phosporyl group between M1P and mannitol. However, Ramos and coworkers showed that neither $EIIBC^{mtl}$ nor $EIIA^{mtl}$ are responsible for the M1Pase activity in *L. lactis* (7). In this study, we clarified that the other phosphoryl transferring protein MtlR also is not responsible for the M1Pase activity. Inducer expulsion, which is a unique mechanism only found in few

Gram-positive bacteria, including *L. lactis*, could be the explanation. Inducer expulsion is the process where intracellular sugar phosphates are dephosphorylated and exported when preferred sugars, like glucose are present (9)(27)(28)(29)(30). In our case, when cells are incubated with glucose, M1P accumulates, is dephosphorylated to mannitol, and transported out of the cell. Interestingly, in *L. lactis* a 10 KDa sugar-phosphatase having a broad substrate specificity was characterized and found to be responsible for inducer expulsion (31)(32). If this enzyme is the M1Pase, we probably could overcome the bottleneck in mannitol production by enhancing its expression, thus avoiding heterologous gene expression (8).

**CCR was relived when cells reach stationary phase**

CcpA is a global transcriptional regulator which is responsible for CCR in Gram-positive bacteria. It functions as a complex with P~Ser-Hpr, and exerts CCR by binding to the *cre* site in front of the sugar transport related genes. In this study, we validate that CcpA serves as a strong repressor for mannitol genes in *L. lactis*. We characterized several mutants adapted to growth on mannitol, and in one of these a mutation was found in the *mtlA* promoter region (C-39T mutant). In the C-39T mutant, the *mtlA* promoter had become more active, both on glucose but especially on mannitol. However, after inactivating the *ccpA* gene, the C-39T promoter displayed a similar activity as the wild type promoter. This indicates that the C-39T mutation had an influence on the functionality of the *cre* site found in this promoter. In stationary phase cells, the activity the mannitol genes appeared to be upregulated around 50-fold higher than that in the exponential phase. This provided an explanation for why the two-step fermentation approach used previously resulted in a high mannitol production in the presence of glucose, which promotes catabolite repression (14). This derepresson could be due to a decreased level of P~Ser-Hpr in the cells, and it has been reported that Hpr mainly exists in the dephosphorylated form in stationary phase cells (25). We speculate that a decreased level of CcpA could also contribute to the observed behavior, however, to clarify this further studies are needed, e.g., where the transcriptome of stationary phase cells is scrutinized.

**The gene coding for a 6S RNA downstream *mtlD* might be influenced by the *mtlD* promoter**

In Figure 3.2, we can clearly see that there is a gene 105 bp after *mtlD*, which was claimed to be a 6S RNA gene by van der Meulen et al. (19). Interestingly, it was shown that the expression of this gene was upregulated by 3 fold after deletion of the *ccpA* gene, and by growing the cells in galactose or cellobiose, repression could also be relieved. Although there is a predicted

promoter and a *cre* site upstream of this gene, there is no predicted transcriptional terminator between this gene and *mtlD*, suggesting that increased expression of the *mtlD* gene could positively affect expression of this 6S RNA gene. In addition, it was also shown that several noncoding RNAs and protein coding genes are regulated by this 6S RNA, and it was deduced to be active when CCR is relieved during stationary phase and/or growth on alternative carbon sources (19).

### *L. lactis* MtlR is different from homologs in other firmicutes

In this study, we substantiated that MtlR indeed is a transcriptional activator, which plays an important role for expression of the mannitol genes, and we characterized an mutant *mtlA* promoter found in a mannitol adapted strain which growing fast in mannitol. The mutated *mtlA* promoter retained an activity of around 40 MU in the absence of mannitol, which appears to be due to less carbon catabolite repression, as the mutation locates to the predicted *cre* site. In *B. subtilis*, MtlR binds to an incomplete inverse repeat sequence upstream of the *mtlA* promoter (5). In *L. lactis*, a similar inverse repeat sequence can also be found upstream of both *mtlA* and *mtlD* (Figure 3.2(b)). Interestingly, these inverse repeat sequences are likely to form rho-independent terminators, as they contain a GC rich harpin following by several Ts. From a protein alignment between studied MtlRs from other Gram-positive bacteria, we found that *L. lactis* MtlR is much more similar to the *S. mutans* MtlR (Table S1). However, in one study it was claimed that MtlR in *S. mutans* was not needed for expression of the mannitol genes, and that this was due to a long insertion in the *mtlR* gene (33). Despite of this, in most other studies of MtlR in firmicutes, it has been shown that MtlR indeed acts as transcriptional activator for the mannitol operon (34)(35)(36)(37)(38)(39). MtlR generally consist of two parts. The first part is the helix-trun-helix Mga domain, which was first characterized in the *S. pyogens* virulence gene regulator Mga, where it is responsible for binding to its DNA target. The second part consist of PRD domains and $EIIA^{Mtl}$-like domains (as well as $EIIB^{Gat}$-like domains in some firmicutes), which regulate the activity of MtlR through their phosphorylation states. In general, it has been found that the PRD1 domain has little effect on the activity of MtlR, whereas the phosphorylation status of the PRD2 domain greatly affects activity, where phosphorylation activates MtlR. Furthermore, dephosphorylation of the $EIIA^{Mtl}$-like and $EIIB^{Gat}$-like domains has positive effects on the activity of MtlR. Interestingly, the *L. casei* MtlR is only regulated by its $EIIA^{Mtl}$-like domain (3). From sequence alignment and domain prediction, *L.lactis* MtlR appears structurally similar to *L. casei* MtlR, which does not have PRD1 and $EIIB^{Gat}$–like domains. However, the histidine in PRD2 domain is conserved in *L. lactis* MtlR (Figure S1,

S2). Moreover, in *B. subtilis*, it was shown that regulation of the mannitol operon mainly takes place via phosphorylation of MtlR, and that CcpA mediated carbon catabolite repression does not play a significant role, as expression of mannitol genes was not significantly affected in a *ccpA* deficient strain (6). Differently, in *L. lactis*, we have shown CcpA is a strong repressor of the mannitol genes, while MtlR seemingly is still active in the presence of glucose. In *G. stearothermophilus* and *L. casei*, phosphorylation in the $EIIA^{Mtl}$-like domain by P ~ $EIIB^{Mtl}$ inhibits the activity of MtlR(3)(40). Several studies have shown that mannitol production can be enhanced by deleting *mtlF*, and it has been concluded that this is due to prevention of re-uptake of the mannitol produced (14)(41)(7). In this study, we detected a four fold higher MtlR activity in a *mtlF* (encoding $EIIA^{Mtl}$) deficient stain, where $EIIB^{Mtl}$ always is in an unphosphorylated state because of the absence of its phosphoryl group donor $EIIA^{Mtl}$. This suggests that the $EIIA^{Mtl}$-like domain in *L. lactis* MtlR might be functional, and that phosphorylation of the $EIIA^{Mtl}$-like domain could negatively affect the activity of *L. lactis* MtlR (Figure 3.7). If so, deleting the *mtlF* gene not only inhibits uptake of mannitol from the medium, but also to enhances the M1PDH activity by activating MtlR, thus stimulating mannitol production in *L. lactis*.

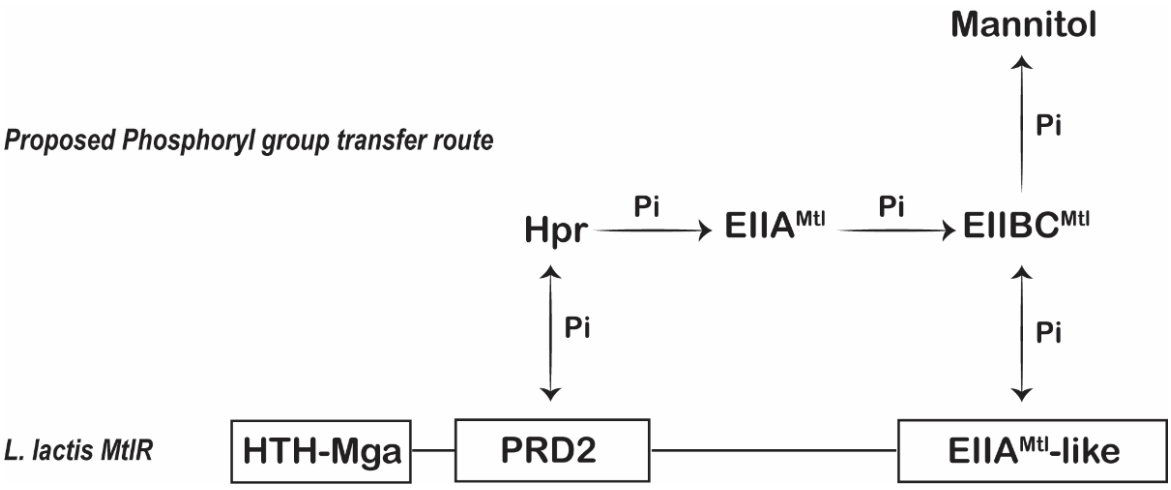


**Figure 3.7. The proposed phosphoryl group transfer route in *L. lactis* MtlR.**

**Transcriptome analysis provide new visions for mannitol operon regulation**

In this study, we have analyzed the expression of genes in *L. lactis* mannitol operon in transcriptional level. The results from transcriptome analysis were highly consistent with what we have seen from the *gusA* reporter gene assay. One exception is that when cells reach stationary phase in mannitol, the expression level of mannitol genes was shown to be decreased in RNA level but increased in protein level. We speculated it because of the protein accumulation. When cells incubated in mannitol, mannitol genes was highly induced even in exponential phase, leading to protein accumulation from the whole fermentation process. Furthermore, the gene just following the mannitol operon, which was characterized to code 6S RNA gene (19), were found to be affected by the expression level of mannitol operon and *ccpA*. 6S RNA is a highly conserved small regulatory RNA, regulating transcription of a large amount of genes by interacting with RNA polymerase (20). Furthermore, 6S RNA was found to be upregulated in stationary phase and play a critical role for cells surviving (21)(22). This make sense that we found the expression of *L. lactis 6S RNA* gene was repressed by *ccpA* but not as tight as what we have seen for mannitol genes. By analyzing the structure of the promoter region of *L. lactis 6S RNA* gene (19), a cre site was predicted to locate preceding the -35 element. In contrast, the location of cre sites in before mannitol genes are overlapped with their -10 elements. This might give an explanation why *ccpA* controls *6S RNA* not tight as it does in mannitol genes. Besides *ccpA*, mannitol operon also affect the expression level of *6S RNA*. Actually, to compare the transcriptome information between AceCR, and AceCF, we found 15 genes in the *L. lactis* genome were changed in their expression level at least for 2 times (data not shown). This finding is pretty novel and may provide another vision that why mannitol was found to be beneficial for *L. lactis* surviving in harsh conditions (23)(24). Moreover, as a central regulator for CCR, unexpectedly, *ccpA* was found to have no big change in its expression level in different condition, even was slightly upregulated when cells growing in mannitol. Since *ccpA* exerts CCR by interacting with P~Ser46-Hpr, we deduced that the derepression of mannitol operon by inducing with mannitol or by shifting cells into stationary phase, were because of the dephosphorylation of P~Ser46-Hpr in these conditions. This make sense because Ser46-Hpr was found to be predominantly non-phosphorylated in the stationary growth phase (25). Thus, we conclude that CCR stage in *L. lactis* were not resulted from the change of expression level of *ccpA* gene.

## 3.6 References


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# Chapter 4. Mannitol production in *L. lactis* with two-stage fermentation

This chapter is partially based on of our work ‘‘Harnessing Adaptive Evolution to Achieve Superior Mannitol Production by *Lactococcus lactis* Using Its Native Metabolism, *Journal of Agricultural and Food Chemistry*, 2020’’ and ‘‘Deciphering the regulation of the mannitol operon paves the way for efficient production of mannitol in *L. lactis, Applied and Environmental and Food Microbiology*, 2021’’.

## 4.1 Abstract

Mannitol production in *Lactococcus lactis* (*L. lactis*) depends on a sufficient activity of the mannitol-1-phosphate dehydrogenase, which is expressed from its own promoter ($P_{mtlD}$). This promoter is regulated by MtlR and CcpA, where the latter regulation is de-repressed in in stationary phase. Thus, to achieve mannitol production we developed a two-stage fermentation setup, where biomass first was accumulated by aerated culturing, followed by a mannitol production phase with limited aeration. By using this approach, 15 mM of mannitol could be generated with a 50% yield from glucose. The strain used, AceF, was severe hampered in NADH generation, Since the only way for NADH regeneration in this strain is through reducing fructose-6-phosphate (F6P), an intermediate derived from glucose, which transported by PTS-PEP system and need phosphoenolpyruvate, ATP shortage will happen when cells under aeration-limited condition. After supplementing ATP by adding arginine, 35 mM mannitol with a conversion ratio of 60% could be achieved. By further overexpressing *mtlD* with cloning plasmid, around 54 mM mannitol could be achieved. To the best of our knowledge, these are the highest mannitol titer as well as the yield produced from *L. lactis* have ever reported. Besides, a huge among of acetoin, around 70 mM could be achieved as the main by-produce from the whole fermentation process. Furthermore, we have firstly confirmed that *L. lactis* could produce mannitol sugars excepted glucose with its native metabolism. Although using glucose as a substrate for producing mannitol is still the most efficient way, we demonstrated a great potential for developing *L. lactis* to produce mannitol from a broad of sugars and their derivates, which could eventually broaden the application for using *L. lactis* as a mannitol producer.

## 4.2 Introduction

Mannitol is a six-carbon sugar alcohol with therapeutic as well as food applications. In the past two decades, the production of mannitol by Lactic Acid Bacteria (LAB) has been studied extensively, especially in heterofermentative LAB (1). In the latter, mannitol is formed from fructose in a one-step reduction process, catalyzed by the mannitol dehydrogenase (MDH). Heterofermentative LAB indeed have a great capacity for producing mannitol, however, mannitol can only be formed when fructose or fructose containing carbohydrates like sucrose are available. Unlike heterofermentative LAB, for mannitol production, homofermentative LAB rely on a mannitol 1-phosphate dehydrogenase, which catalyzes the interconversion of fructose 6-phosphate (F6P), a glycolytic intermediate, and mannitol 1-phosphate.

Homofermentative LAB, in principle, therefore are able to produce mannitol from a broader range of carbohydrates than heterofermentative LAB.

The homofermentative LAB *Lactococcus lactis* (*L. lactis*), with its long history of safe use in the food industry, is a good candidate as a mannitol producer. In the recent past, there have been several attempts at turning *L. lactis* into an efficient mannitol producer. *L. lactis* does not produce mannitol naturally, and the reason for this is low inherent mannitol 1-phosphatase (M1Pase) and mannitol 1-phosphate dehydrogenase (M1PDH) activities. However, mannitol production has been observed in lactate dehydrogenase (*ldh*) deficient *L. lactis* strains (2), and can be enhanced by heterologous expression of M1Pase and M1PDH (3).

We previously reported efficient production of mannitol from glucose, with a 60% yield (theoretical max. is 66.7%), using *L. lactis* strains blocked in $NAD^+$ regeneration, which had been adapted to aerobic growth on mannitol (Chapter 2). Characterization of these strains revealed the presence of mutations enhancing the expression of the genes involved in mannitol metabolism. Expression of these genes is tightly controlled by the mannitol regulator MtlR (*mtlR*), and the global transcriptional regulator CcpA, where regulation by the latter is relaxed in stationary phase cells (Chapter 3). Furthermore we found that the enzyme IIA phosphotransferase system component ($EIIA^{mtl}$), encoded by *mtlF* also has a regulatory role, as $EIIA^{mtl}$ can phosphorylate MtlR, which reduces its activity, and that carbon catabolite repression can be partially relieved when cells enter the stationary phase.

In this study, based on our previous work, we try to establish an efficient and food-grade approach for producing mannitol using *L. lactis*. We explore the potential of stationary phase cells for producing mannitol, and investigate the effect of inactivating *ccpA*. Furthermore, we investigate the influence of ATP supply, redox status and the sugar being metabolized.

## 4.3 Methods

### Bacterial Strains and Growth Conditions

AceF, which is derived from *Lactococcus lactis* subsp. *cremoris* MG1363, and its derivatives were used in this study (described in Table 4.1). *L. lactis* strains were cultivated in M17 broth supplemented with 1% glucose (Sigma Aldrich) at 30°C, with shaking at 240 rpm. When needed, 5 μg/ml tetracycline or 10 μg/ml chloramphenicol was used for selection, and 2 μg/ml tetracycline and 4 ug/ml chloramphenicol was used in physiological growth experiments. The optical density of cell suspensions was measured using a spectrophotometer (Shimadzu) at 600

nm. Resting cells preparation and two-stage fermentation were performed as described previously (4).

**Molecular techniques**

Electrocompetent cells were prepared using the protocol of Holo & Nes (5), using GM17 medium containing 2% glycine and 0.25 M sucrose. The medium was filtered (not autoclaved). Electroporation was performed as previously described by Holo and Nes using a MicroPulser Electroporator (Bio-Rad) (5). Phusion DNA Polymerase (Thermofisher Scientific) was used for PCR amplifications. Zyppy plasmid miniprep kit (Zymo Research) was used for extracting the plasmids from *L. lactis* after a pre-treatment with 20 mg/mL lysozyme (Fluka, 7,000U/mg) at 37°C for 2 hours. FastDigest restriction enzymes were obtained from Thermofisher Scientific.

- Overexpression of *pta*

First, a 1768 bp fragment containing the entire *L. lactis pta* gene and its flanking sequence was amplified using primers 5'- GCGTGACTTAATGGATATCA -3' and 5'- ATCATTGCTGCTGTAGATAAT -3'. For the second PCR reaction, a pair of primers for amplifying a 1458 bp fragment containing restriction enzymes sites (SacI and BamHI), the native *pta* promoter and the whole coding region of *pta gene* were used by forward primer 5'- AGTCTGAGCTCGCGTGACTTAATGGATATCA -3' together with the following reverse primer 5'- AGTCTGGATCCTTATTTTAAAGCTTGAGCAGC -3'. All primers are ordered from IDT company. The amplified fragments were digested with restriction enzymes and inserted into pCI372 and the constructs introduced into AceF and verified by Sanger sequencing (Macrogen).

- Overexpression of *gapB* gene

The gapB gene from IL1403 was amplified using a sequence containing a synthetic promoter as forward primer: ACGACTAGTGGATCCATNNNNNAGTTTATTCTTGACANNNNNN-NNNNNNNNTGRTATAATANNNGAGTAAGTTAAATTGTTAACTTAG (N is 25% A, G, C, T and R is 50% A/G), and CTCTACATGCATGTTTTTATACCGTTAAAATCGG as reverse primer. The products were double digested by SpeI/NsiI and ligated in pCI372 (6). Sequencing the promoter region giving CTAGTGGATCCATGGGACAGTTTAT-TCTTGACAAGAGTCGAGGATGGTGATATAATAGGG, which resulted in 3.4 times of GAPDH activity higher than that in wild type MG1363. This plasmid was prepared and provided by Christian Solem.

**Quantifying mannitol production by High-Performance Liquid Chromatography (HPLC)**

For quantifying glucose and mannitol, we used an HPLC equipped with a BioRad Aminex HPX-87H column (BioRad) and a RI-101 detector (Shodex). The mobile phase consisted of 5 mM $H_2SO_4$ at a flow rate of 0.5 ml/min. The column oven temperature was set to 60°C. The samples for HPLC analysis were filtered using 0.22 μM filters (Labsolute) and loaded to HPLC immediately after sampling at appropriate cells density.

**β-glucuronidase assays**

Strains were cultured in M17 broth supplemented with 1% of the indicated sugar and 2 μg/ml tetracycline. Cells were harvested after reaching $OD_{600}$=1.0 or in the stationary phase ($OD_{600}$=4.0), and quenched on wet ice followed by centrifugation at 4°C. Harvested cells were washed in ice cold PBS and resuspended in ice-cold Z buffer (0.06 M $Na_2HPO_4$, 0.04 M $NaH_2PO_4$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH=7). Cells were appropriately diluted and the cell density (optical density) was measured at 600 nm. For permeabilization, 12.5 μl 0.1% SDS and 25 μl $CHCl_3$ were added, and the suspension vortexed vigorously. After equilibrium at 30°C for 5 min, 100 μl of 4 mg/ml PNPG was added, and the time needed for developing a yellow color recorded. To stop the reaction 800 μl $Na_2CO_3$ was added. Absorbance at 420 nm and 550 nm were measured. Promoter strength was calculated as $1000*(OD_{420}-1.75*OD_{550})/(time*OD_{600}*ml\ sample)$ and describe as Miller units.

**Table 4.1. Overview of strains and plasmids used in this study**

| Strain and plasmid | Relevant genotype | Source or reference |
|---|---|---|
| **Strains** | | |
| **AceF** | Mannitol adapted MG1363 *Δ*$^{3}$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA, ΔmtlF* | Chapter 2 |
| **AceCF** | Ace001-M *ΔmtlF, ΔccpA* | Chapter 3 |
| **Ace001-mtlD** | *mtlD* overexpressed Ace001, 20%TPI promoter | Chapter 3 |
| **AceF-gapB** | *gapB* overexpressed AceF | |
| **AceF-pta** | *pta* overexpressed AceF | This study |
| | | |
| **Plasmids** | | |
| **pTD6** | pTD6 A derivative of pAK80 containing a *gusA* reporter gene, Tet$^{R}$ | (7) |
| **pCI372** | *E. coli*/*L. lactis* shuttle vector, Cam$^{R}$ | (6) |
| **pCI372-gapB** | pCI372 with a synthetic promoter preceding *gapB* gene | Provided by Christian Solem |
| **pCI372-pta** | pCI372 with 20% TPI promoter preceding *pta* gene | This work |
| **pTD6-mtlD** | pTD6 with 20% TPI promoter preceding *mtlD* gene | This work |
| **pPmtlD** | pTD6 with *mtlD* promoter preceding *gusA* reporter gene | Chapter 3 |
| **pPmtlA$^{mut}$** | pTD6 with mutant *mtlA* promoter preceding gusA reporter gene | Chapter 3 |

**Two-stage fermentation**

AceF was pre-cultured aerobically in M17 containing 1% of the indicated sugar until reaching the desired cell density. Culture medium containing cells was immediately distributed into different volumes, either 25 ml, 50 ml, 75 ml or 100 ml in 100 ml flasks. Selected cultures were supplemented with 35 mM arginine (pH = 7.0) and additional indicated sugars, and shaken in water bath at 30°C, either at 20 or 60 rpm. As controls, vigorously aerated cultures (25 ml medium in 250 ml flask, 240 rpm) and non-aerated (full-filled medium in 100 ml flask, static) cultures were included. Samples were withdrawn at particular time points and analyzed by HPLC.

## 4.4 Results

### 4.4.1 Using a two-stage fermentation setup to produce mannitol

In our previous study (Chapter 2), we found that Ace001M and AceF (MG1363 *Δ*$^{3}$*ldh*, *Δpta*, *ΔadhE*, *ΔbutBA, ΔmtlF*) were able to produce small amounts of mannitol from glucose with high yield (60%) under static conditions. In AceF, gene encoding Enzyme IIA of the mannitol PEP phosphotransferase system (*mtlF*), has been deleted, which prevents inactivation of MtlR by phosphorylation, which should make this particular strain well suited for mannitol production. Under normal circumstances, the presence of glucose, represses expression of the genes needed for producing mannitol. Nevertheless, this repression could be relaxed when cells reach stationary phase (Chapter 3). Furthermore, mannitol production consumes ATP, and it is possible that the ATP supply is insufficient to support both growth and mannitol production. To address these concerns, we designed a two-stage fermentation setup for producing mannitol using *L. lactis* AceF: 1) biomass accumulation, 2) high cell density mannitol production. In the first stage, *L. lactis* is cultured aerobically until cells reach the stationary phase. In the second stage, aeration is terminated, and mannitol production is the only way for the cells to regenerate $NAD^+$. In the second stage, growth is drastically reduced, possibly because ATP is spent on mannitol production, making less ATP available for growth. One benefit of using stationary cells for producing mannitol is that carbon catabolite repression is relieved in these cells (Chapter 3). To test whether the cells were starved for ATP, we examined the effect of adding arginine, as ATP is generated when arginine is metabolized.

To determine which conditions are best for mannitol production, a series of experiments were set up, more specifically investigating the effect of aeration, fermentation volume and arginine. Three types culturing were investigated: 1) ***static culturing***, i.e. where no efforts were made to

facilitate aeration (slow diffusion of oxygen into the cultures is feasible), 2) ***culturing with slight aeration (semi-anaerobic)***, i.e. either low shaking speed (20 rpm) or "high" shaking speed (60 rpm), and 3) ***full aeration***, where the shaking speed was very high and where the oxygen supply was not limiting (240 rpm).

**Mannitol production where the cell density in stage 2 is relatively low ($OD_{600}$ = 0.5)**

As shown in Figure 4.1a, when starting from a low cell density, $OD_{600}$ of only 0.5, the amount of mannitol formed without arginine added ranged from 3.4-11.8 mM, depending on whether the cultures were aerated or not, and ranged from 5.0-14.5 mM when arginine was supplied. The yield from glucose ranged from 14-58%. Supplying oxygen had a beneficial effect on cell growth and mannitol production, however, a negative effect on mannitol yield. Interestingly, small amounts of mannitol (1.62-1.81 mM) were also detected, when AceF was cultured under aerobic conditions. In general, when arginine was added, this resulted in an increase in the final pH of the cultures. With arginine the pH ranged from 7.16 to 7.74, whereas the range was 6.65 to 6.94 without arginine added, and this was most likely due to the $NH_4^+$ formed when arginine is metabolized.

**Mannitol production where the cell density in stage 2 is relatively high (OD600 = 4.5)**

When a higher initial cell density was used ($OD_{600}$ of 4.5, stationary cells), higher titers of mannitol, ranging from 8.24-15.0 mM without arginine, and from 27.3 mM to 33.4 mM with arginine added. The mannitol yield ranged from 41-63% (Figure 4.1c). Interestingly, when compared with the low cell density experiments, a more stable mannitol titer was observed for the aerated cultures, however, supplying oxygen in this case still had a negative effect on the mannitol yield. To assess whether the ATP supply was limiting mannitol production, the [ATP]/[ADP] ratio was determined. By adding arginine it was possible to increase the [ATP]/[ADP] ratio, and maintain it better over the course of the fermentation (Figure 4.2).

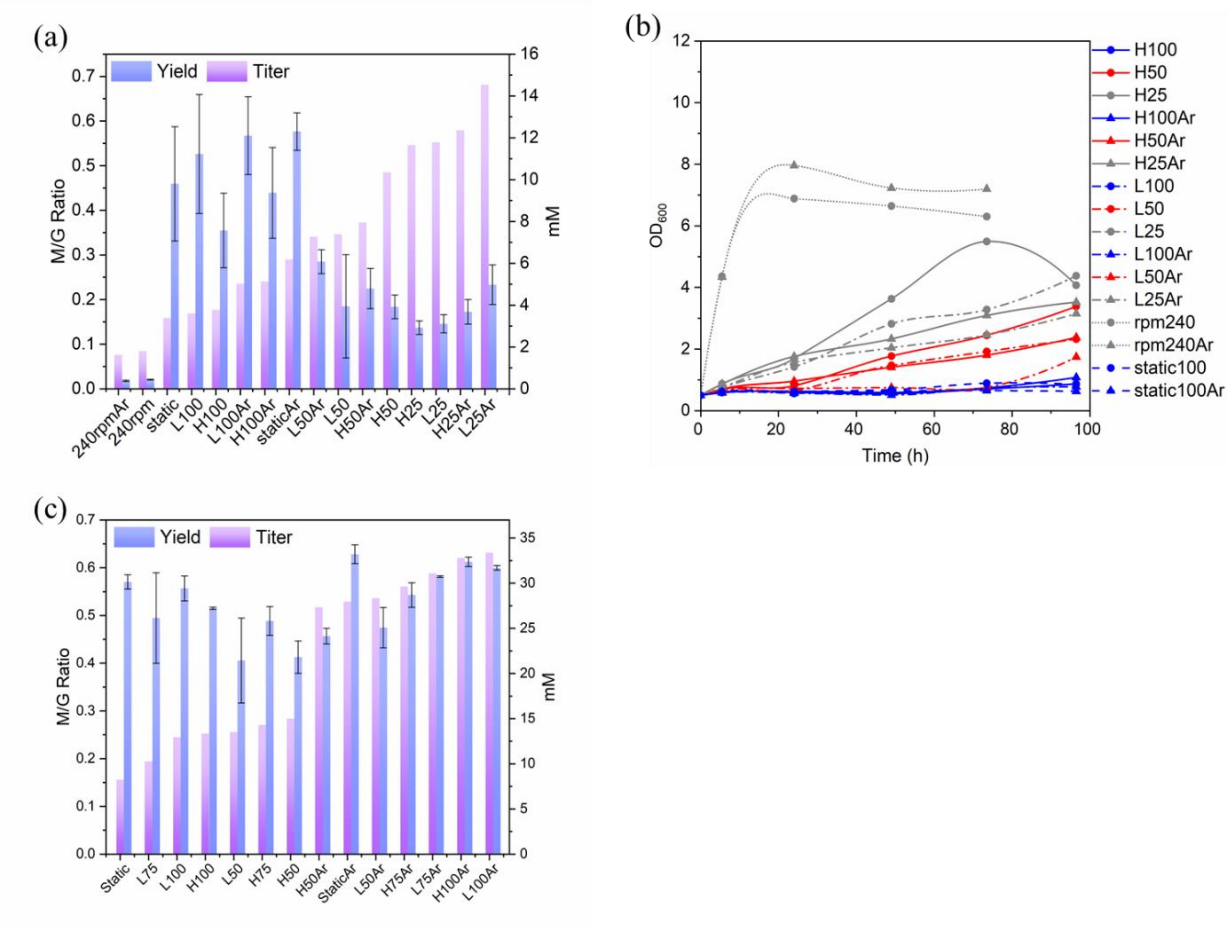


**Figure 4.1. Comparison of the effect of aeration and arginine supplementation on mannitol production in AceF.** All data were collected within 96 hours from specific cultivation. (a) Yield and titer of mannitol achieved in AceF cultures initiated at a low cell density of $OD_{600}$ = 0.5. (b) Growth curves for AceF cultures initiated at a cell density of $OD_{600}$ = 0.5. (c) Yield and titer of mannitol achieved in AceF cultures initiated at a high cell density of $OD_{600}$=4.5. H indicates high shaking speed (60 rpm) and L indicates a low shaking speed (20 rpm); 240rpm indicates that 25 ml medium in 250 ml flask shaking with 240 rpm Static refers to cultures not being actively aerated, slow diffusion of oxygen possible; Numbers after H/L indicate the volume of medium used in 100 ml flasks; Ar after numbers, indicates addition of 35 mM of arginine. Yields are calculated based on data collected at different time points during the experiment, and shown with standard deviations.

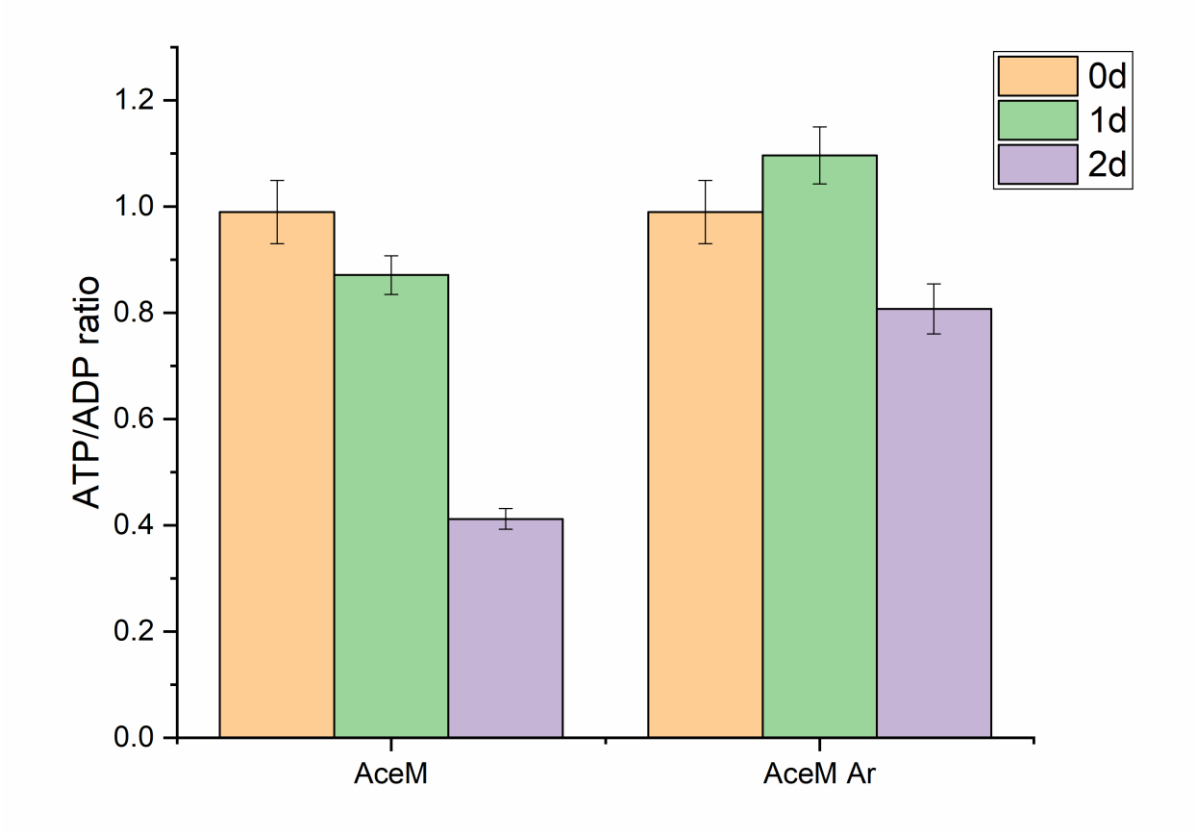


**Figure 4.2.** Intracellular ATP/ADP ratio in AceF with or without supplementing arginine using two-stage fermentations. . Results were obtained from high-density cells (OD600=4.5). Ar stands for arginine was added. All treatments were duplicated.

### 4.4.2 Attempts at enhancing mannitol production by metabolic engineering

Mannitol production using strains hampered in $NAD^+$ regeneration in combination with a two-stage fermentation setup appears to have great potential. It is likely that mannitol production can be improved further by employing different metabolic engineering strategies. Our previous research (Chapter 3) revealed that it was possible to increase expression of the mannitol genes by deleting *ccpA*, and we decided to test whether this could be beneficial for mannitol production. Arginine had a clear beneficial effect on mannitol production, which probably was linked to ATP supply. We therefore decided to restore the ability to produce acetate in AceF, as acetate formation results in formation of additional ATP, and this was accomplished by overexpressing *pta*, encoding phosphotransacetylase. We also speculated that the [NADH]/[$NAD^+$] ratio could have a negative impact on mannitol production, as a high [NADH]/[$NAD^+$] ratio is known to affect glycolysis negatively through its effect on

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (8). To address this we overexpressed GAPDH, which is encoded by *gapB*.

**Overexpressing *mtlD* and restoring acetate formation is beneficial for mannitol production**

By using our two-stage fermentation setup, once the tested strains reach their stationary phase (OD600=4.0-4.5), 2% glucose and 35 mM arginine were added, with limited aeration (20 rpm) performed. As shown in Figure 4.3, for all strains tested, mannitol productivity was greatest in the first 24 hours of the fermentation, and subsequently decreased gradually. The reference strain, AceF, was able to generate 35.0 mM mannitol during 5 days, in the presence of arginine. By overexpressing *mtlD* in AceF, the mannitol titer increased to 53.9 mM, which is the highest mannitol titer ever reported for *L. lactis*. The effect of deleting *ccpA* was somewhat unexpected, and the mannitol titer only reached 35.1 mM, which is similar to AceF. CcpA is involved in activating the expression of the *las* operon and other glycolytic genes (9), and it is thus likely that deletion of *ccpA* will hamper glycolysis, and this will have a negative effect on mannitol production. Restoring acetate formation by introducing the *pta* gene resulted in 22.23 mM acetate formed as byproduct and 32.8 mM mannitol.

Besides, no positive effect on mannitol production was observed by overexpressing GAPDH gene which resulted in 28.3 and 25.4 mM mannitol respectively. The yield of mannitol for all tested strains were similar, ranging from 53.9% to 60.3%, with one exception, AceF-GAPDH, which resulted in 46.6% yield. In addition, all strains produced a large amount of acetoin, ranging from 57.8 mM to 73.7 mM, where the strain overexpressing GAPDH produced the most, suggesting an enhanced glycolysis flux in its growing. Intriguingly, when cells shifted into stationary phase and incubated under anaerobic condition, acetoin production was much slower and yielded with a conversion ratio of 10%-30% in this period. HPLC analysis of fermentation samples revealed several peaks representing unknown compounds.

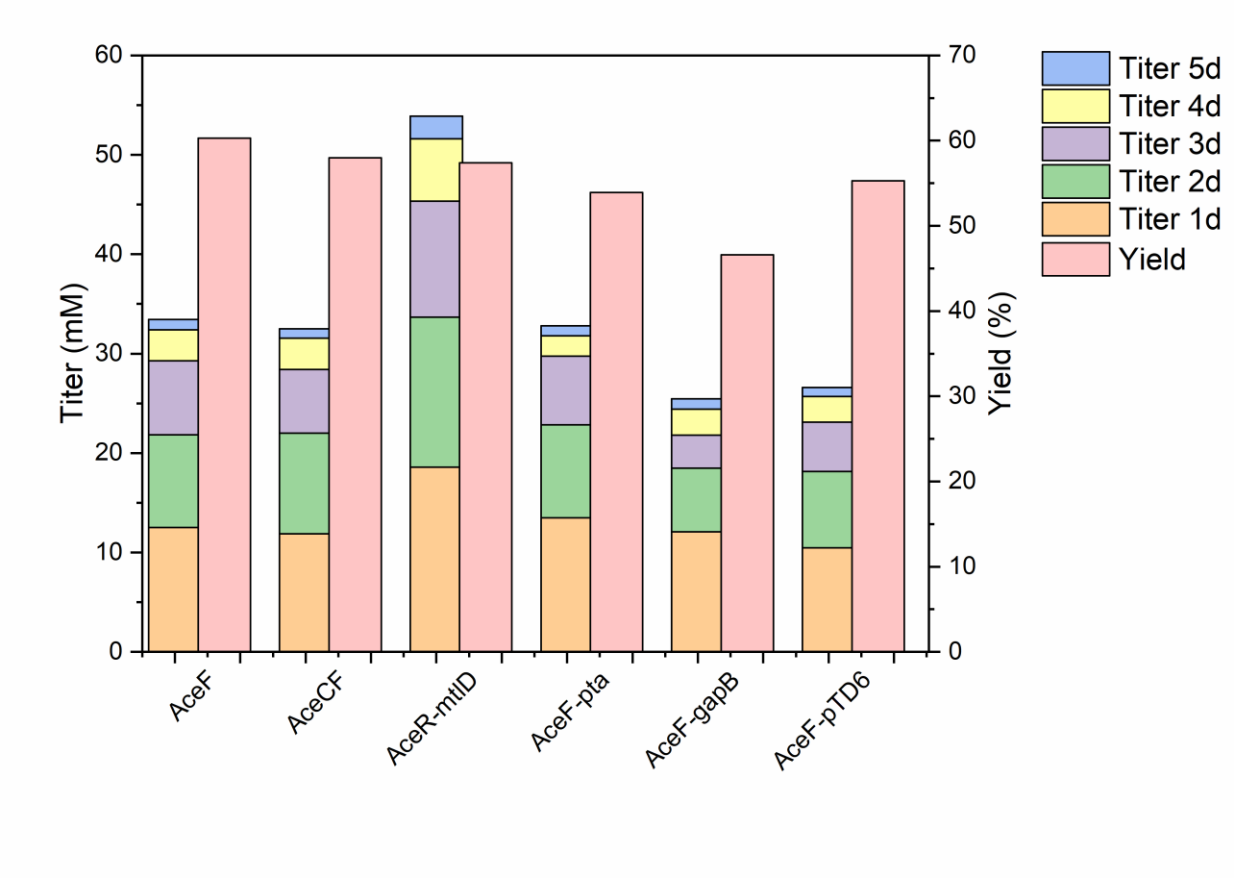


**Figure 4.3. Mannitol production from stationary *L. lactis* with different genetic background.** 100 ml Stationary cells (OD=4.0 or 4.5) in 100 ml volume flask were incubated with 2% glucose and 35 mM arginine under limited aeration (20 rpm). Mannitol produced in each day were indicated with different color; Yield of produced mannitol from stationary phase were indicated.

**Table 4.2. Mannitol production in *L. lactis* with different genetic background by using two-stage fermentation**

| Strains | Mannitol (mM) | Yield (%) | Acetoin (mM) | Yield (%) |
|---|---|---|---|---|
| AceF | 35.0 | 60.3 | *61.4 | 31.8 |
| AceCF | 35.1 | 58.0 | *60.7 | 25.7 |
| AceR-mtlD$^{\uparrow}$ | 53.9 | 57.4 | *72.2 | 25.3 |
| AceF-pta$^{\uparrow}$ | 32.8 | 53.9 | *57.8 | 11.3 |
| AceF-gapB$^{\uparrow}$ | 25.4 | 46.6 | *73.7 | 14.5 |
| AceF-pTD6 | 26.6 | 55.3 | *63.8 | 26.4 |

**Note:** * stands for acetoin produced from the whole fermentation process (including growing phase).

### 4.4.3 Mannitol production from different sugars

*L. lactis* could produce mannitol base on its intracellular M1PDH and M1Pase activity, which initiates with the reaction from reducing F6P to M1P. Since F6P is a common intermediate in glycolysis process, *L. lactis* is considered as a mannitol producer that could produce mannitol from different sugars. However, no report could be found for using *L. lactis* to produce mannitol from other sugars than glucose. To test this, three common sugars fructose, maltose and galactose were used for accessing the ability of *L. lactis* in producing mannitol besides using glucose.

As shown in Figure 4.4, by using two-stage fermentation, *L. lactis* was able to produce mannitol from all sugars we have used. Using fructose as substrate resulted in higher mannitol titer, and using galactose as substrate resulted in higher yield. When using AceF as mannitol producer, 18.2 mM (26.5%, yield), 12.7 mM (34.7%), 15.3 mM (46.5%) mannitol were produced from fructose, maltose and galactose respectively. Deleting *ccpA* gene in AceF resulted in enhanced mannitol production from fructose and galactose, but resulted in weakened mannitol production from maltose. By using AceCF, 22.2 mM (43.7%), 3.97 mM (29.5%), 15.8 mM (50.0%) mannitol were produced from fructose, maltose and galactose respectively.

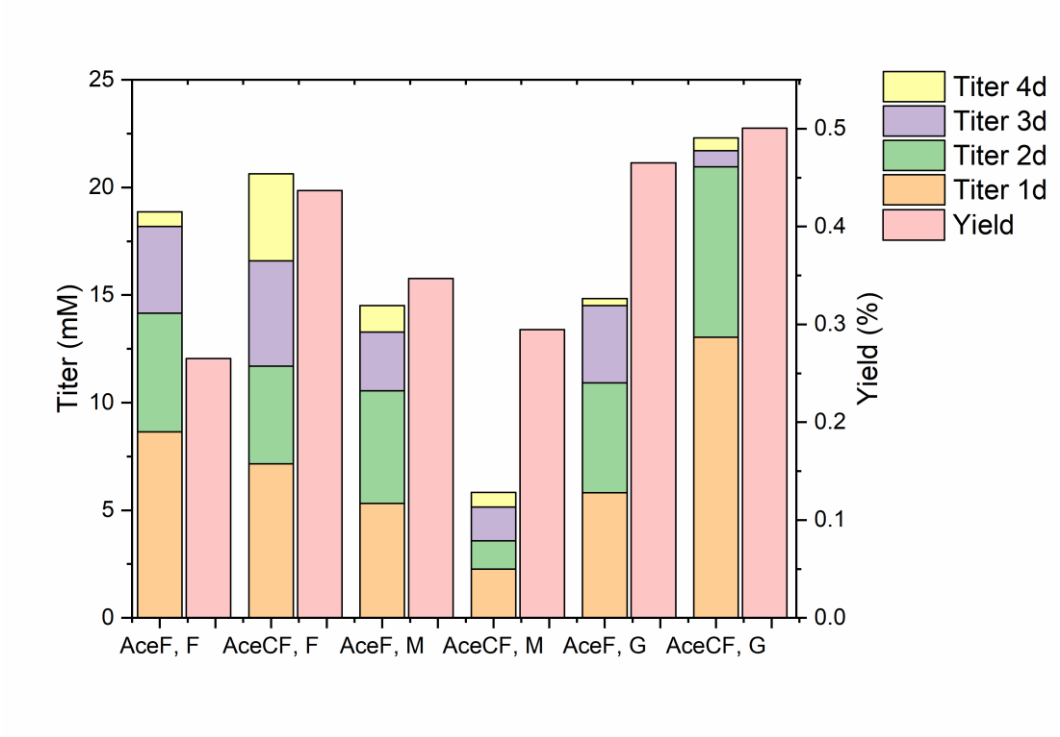


**Figure 4.4. Mannitol production by using stationary *L. lactis* from different carbon resources.** 100 ml Stationary cells (OD=4.0 or 4.5) in 100 ml volume flask were incubated with 2% indicated sugars and 35 mM arginine under limited aeration (20 rpm). Mannitol produced in each day were indicated with different color; Yield of produced mannitol from stationary phase were indicated.

**Table 4.3. Mannitol production in *L. lactis* from different sugars by using two-stage fermentation**

| Sugars | Strains | Mannitol (mM) | Yield (%) | Acetoin (mM) | Yield (%) |
|---|---|---|---|---|---|
| Fructose | AceF | 18.2 | 26.5 | *41.4 | 10.9 |
| Fructose | AceCF | 22.2 | 43.7 | *43.9 | 14.8 |
| Maltose | AceF | 12.7 | 34.7 | *57.4 | 0 |
| Maltose | AceCF | 3.97 | 29.5 | *47.9 | 0 |
| Galactose | AceF | 15.3 | 46.5 | *50.5 | 18.0 |
| Galactose | AceCF | 15.8 | 50.0 | *63.0 | 13.3 |

**Note:** * stands for acetoin produced from the whole fermentation process (including growing phase).

## 4.5 Discussion

### Two-stage fermentation based on the M1PDH dependent mannitol production

In *L. lactis*, mannitol production highly depends on the expression of mannitol operon, which is subjected to CCR (Chapter 3). Interestingly, we found the CCR could be partially relieved by shifting cells into stationary phase. In attempt to improve mannitol production from glucose, we developed a two-stage production setup, where we firstly accumulated biomass and subsequently used this biomass for producing mannitol (Figure 4.5). By using this method, we achieved 6.1g/L mannitol produced from glucose with a 60% mannitol conversion ratio from a food-grade strain, AceF. Moreover, to bypass the CCR by overexpressing its *mtlD* gene in this strain, 9.8 g/L mannitol with a conversion ratio of 57% could be obtained. To the best of our knowledge, this is the first example of *L. lactis* secreting mannitol, without using resting cells nor relying on any foreign genes. Furthermore, the mannitol titer and yield obtained from this study were also the highest value have ever reported from *L. lactis* this far, where the latter one is very close to the theoretical value 66.7%. An advantage of the approach used here, where acetoin is generated, is that the need for pH control is obviated. Furthermore, during the whole process of the two-stage fermentation, a large amount of acetoin (60.7mM to 73.7 mM) was detected as the main by-product. By deleting the *ccpA* gene, which resulted in high constitutive expression of the *mtlD* promoter. Unfortunately, we found that both titer and yield of the produced mannitol did not change much when deleting *ccpA*. One possible explanation is that glucose metabolism is hampered to such an extent that the beneficial effect coming from overexpressing the mannitol genes is lost. In *L. lactis*, when *ccpA* is deleted, glycolysis is hampered (10)(11)(12). Since PTS uptake of glucose requires phosphoenolpyruvate (PEP), a glycolytic intermediate, this will have a direct effect on mannitol production, which is what we indeed observed. It appears that a more promising strategy for relieving carbon catabolite repression would be to inactivate the CcpA binding site upstream *mtlA* and *mtlD* without reducing the activities of these promoters, which perhaps could be accomplished by classical mutagenesis followed by screening. Furthermore, we tested whether overexpression of *mtlD*, the mannitol-1-phosphat dehydrogenase, would enable anaerobic growth and mannitol production, however, this was not the case. It appears that the mannitol-1-phosphatase is a bottleneck in mannitol production, and irrespective of a high mannitol-1-phosphate dehydrogenase activity, anaerobic $NAD^+$ regeneration will remain hampered as long as the phosphatase activity is limiting. We also speculated whether the cells could be starving for ATP, since mannitol production is ATP demanding. To ameliorate the latter, we supplemented

the cell suspensions with arginine, that can be metabolized via the arginine deiminase pathway leading to ATP formation (32). This indeed had a beneficial effect, however, mainly in works in stationary cells. Thus, boosting the inherent mannitol-1-phosphatase activity, e.g. by mutagenesis, thus appears to be a first priority if a successful mannitol production in *L. lactis* is to be established.

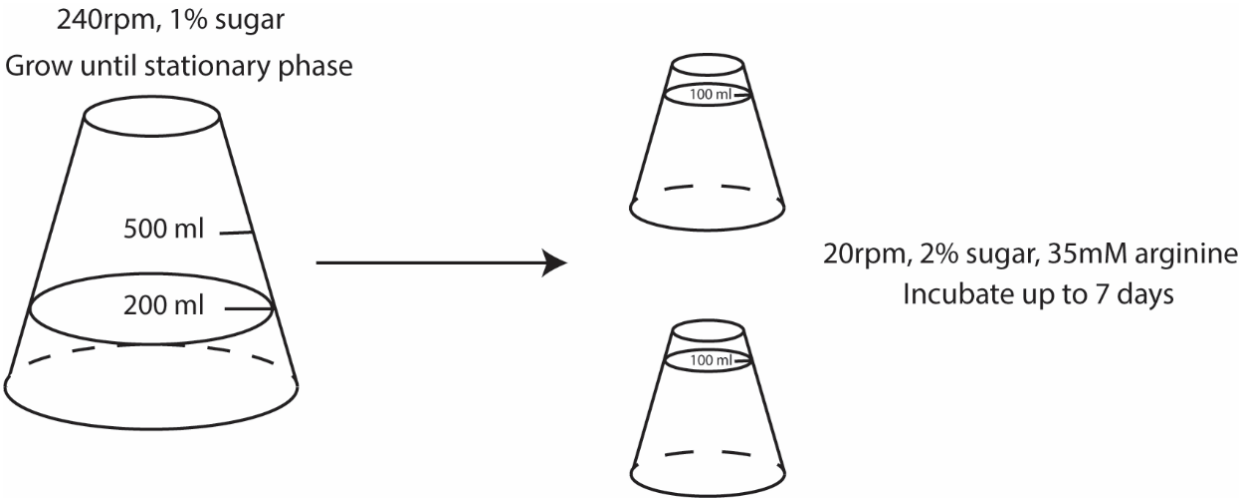


**Figure 4.5. Optimized two-stage fermentation procedure.**

**ATP supplying plays important roles in mannitol production**

In other studies, both the mannitol-1-phosphate dehydrogenase and the mannitol-1-phosphate phosphatase have been shown to be essential for obtaining efficient mannitol production, and over-expression of only one of these enzymes does not lead to high titers in growing cells (3). After introducing *Eimeria tenella mtlP* and over-expressing *mtlD*, *L. lactis* could produce mannitol efficiently, with more than 40% yield of mannitol under growing conditions (3)(13). In our study, mannitol production was also observed from strains hampered in $NAD^+$ regeneration, but all these strains grew poorly. We speculated that the reason for the slow growth was due to inhibition of glycolysis, since glycolysis can be inhibited by a high $NADH/NAD^+$ ratio, which leads to slower ATP formation and hampers growth (14)(15). In AceF cells, formation of one acetoin leads to formation of two ATP and two NADH, and to achieve redox balance, 2 mannitol could be formed for each acetoin formed. Since formation of two moles of mannitol would consume the ATP formed via acetoin production, there is no ATP left for growth or maintenance. In *L. lactis*, arginine is catabolized through the arginine

deiminase pathway, generating $NH_3$, $CO_2$ and ATP (16). To alleviate the NADH burden and provide extra ATP to the cells, we attempted to optimize the fermentation by aeration and supplying arginine. We found that aeration had a beneficial impact on mannitol titer, however, the conversion ratio decreased, due to some of the NADH being oxidized by the NADH oxidase. When arginine was added we observed higher mannitol titers and mannitol conversion ratios. Cells supplemented with arginine were shown to have higher ATP/ADP ratio and slower decline over time. Nevertheless, cells were still struggling to grow under anaerobic condition. We speculate that the extra ATP supplied by arginine metabolism, would allow cells to grow slowly, but the flux would still be limited by a bottleneck in glycolysis. By overexpressing GAPDH, of which the enzyme activity shown to be inhibited in high NADH circumstances, an enhanced cell growth and mannitol production was observed (Figure S7(a)), however, it did not help working on stationary cells. In another hand, extra ATP also could be supplied from acetate production. In this study, we indeed observed higher mannitol production after recovering PTA activity in cells (Table 4.2). However, mannitol yield were shown to be decreased and cells were still grow slow in growing condition.

**F6P pool is an essential for mannitol production in *L. lactis***

Several studies has shown LDH deficient *L. lactis* could produce mannitol in resting condition but not in growing condition (17)(18). It was deduced because F6P pool is too low to generate M1P under growing condition, due to the fast glycolytic flux. This point was supported by using a strain with reduced PFK activity, where an enhanced mannitol production was detected (17)(3). In this study, by using the cells severely hampered in NADH regeneration, we detected around 12 mM mannitol could be produced in slow groing cells (Table S6). However, these cells actually grew much slower than the strains used in other works, and therefore should be considered as growing-limited cells, which might lead to an increased F6P pool due to the hampered growth. Besides F6P pool, the M1Pase activity also a limitation for mannitol production in *L. lactis*. LDH deficient strain could produce mannitol by enhancing both M1Pase and M1PDH activity in growing condition, but did not work by enhancing only one of them. This suggesting an enhanced M1PDH activity help generating M1P even from low F6P pool in the fast growing cells, but still unable to produce mannitol base on the inefficient native M1Pase activity. In addition, the M1P generate from F6P would not accumulated in cells because M1PDH is a bidirectional enzyme and also the F6P pool is low. Although intracellular M1Pase activity could be detected from *L. lactis*, to date, we do not know the coding gene of this enzyme and it likely not destined to converting M1P to mannitol, and there might have

other competitive substrates particularly in growing condition. Due to this, the native M1Pase in *L. lactis* will never generate mannitol if the M1P pool is not high enough. In another word, a low M1Pase activity might support mannitol production only when F6P pool is high, which could be achieved by reducing PFK activity or using resting cells. In this study, we detected a large amount of mannitol could be produced by cells shifted into stationary phase. Regardless the induced expression of *mtlD* gene in this condition, it also likely resulted from an increased F6P pool in stationary cells.

**Great potential for using *L. lactis* producing mannitol from a broad repertoire of sugars**

A big advantage for using *L. lactis* as mannitol producer is that *L. lactis* could theoretically produce mannitol from all sugars undergo glycolysis pathway. Since mannitol production in *L. lactis* strongly relies on the expression level of mannitol operon, which is tightly controlled by carbon repression metabolism. When cells incubating with sugars except glucose, carbon repression could be relaxed (Chapter 3) and therefore beneficial for mannitol production. In this study, three common sugars, i.e. fructose, maltose and galactose were used for testing mannitol production in *L. lactis*. As we have shown, all the three sugars could be used for producing mannitol, but unexpectedly, they are less efficient than glucose. We deduced that the possible reason about this lies in the transport of these three sugars are not as efficient as glucose even when CCR has relieved. Furthermore, when cells were incubated with galactose and fructose, faster mannitol production was detected than using maltose. It probably because fructose and galactose need shorter biochemical routes to be converted into F6P than that for maltose. Nevertheless, as the best of our knowledge, this is the first example that using *L. lactis* to produce mannitol from sugars except glucose by using its native metabolism. This validated a great potential for using *L. lactis* to produce mannitol from a broad repertoire of sugars and their derivates, such as some cheap substrates from industrial waste, which could broaden the application for using *L. lactis* as mannitol producer.

## 4.6 Reference


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# Chapter 5. Conclusions and future directions

Although mannitol production from *L. lactis* has been attempted in last two decades, most of the achievements were relying on the introduction of foreign genes, which would limit their applications in foods. In our aim to develop a food-grade mannitol producing *L. lactis*, in the current study, we have systematically characterized gene expression and regulation of the mannitol operon, which was shown to be critical for mannitol production in *L. lactis*. In addition, by combining strategies such as laboratory evolution, metabolic engineering, and fermentative optimization, 6.1 g/L mannitol with 60% yield (theoretical max. 66.7%) from glucose could be achieved from a food-grade strain, AceF. By further overexpressing *mtlD* gene, 9.8 g/L mannitol with 57% yield could be achieved. Both the yield and titer were the highest value have ever reported for *L. lactis*. A large amount of acetoin (over 6.2 g/L) was produced as the main by-product during these fermentations.

We also showed that by using our food-grade strains, AceF or AceCF, 4.0 g/L, 2.3 g/L, and 2.9 g/L mannitol could be produced from fructose, maltose and galactose, respectively. Taken together these studies have validated the great potential for using *L. lactis* as mannitol producer and provided novel findings that can guide the further development of food-grade *L. lactis* strains with even better mannitol production in the future.

## 5.1 New findings from this study

- The mannitol operon has two transcriptional units. The first one consist of three genes *mtlA*, *mtlR* and *mtlF*, while the other only have one gene *mtlD*. Both of these two transcriptional units were found to be tightly regulated by repressor *ccpA* and activator *mtlR* together. *mtlF*, the gene coding mannitol specific PTS-EIIA protein, also provides a negative feedback on the expression of mannitol operon. The CcpA binding site in the mannitol operon could be mutated by adapting cells in mannitol, resulting in lower carbon catabolite repression (CCR) response. In addition, CCR could also be alleviated by shifting cells into stationary phase, but this is not because of a lower *ccpA* expression level in stationary phase due to the RNA sequencing results. By inactivating both *ccpA* and *mtlF* together, the mannitol operon become permanently activated. Furthermore, the expression of the 6S RNA coding gene, located downstream of the *mtlD* gene, is shown to be affected by the expression status of mannitol operon.

- The mannitol production in *L. lactis* is highly depend on the expression of the mannitol operon, which contains *mtlD*, the key gene for mannitol production. Strategies that alleviate CCR response in the mannitol operon, such as mutating cre sites by laboratory evolution, inactivating *mtlF*, or shifting cells into stationary phase result in positive effects on mannitol production in *L. lactis*. In contrast, deleting ccpA gave only limited effects on mannitol production.
- The yield of mannitol could be strongly enhanced by restricting NADH flux in cells. Nevertheless, the hampered NADH regeneration would result in ATP shortage and hampered glycolytic flux. In this case, supplying cells with extra ATP by either adding arginine to the medium or restoring the *PTA* gene, leads to an enhanced mannitol production in *L. lactis*.
- In addition to glucose, other sugars like fructose, maltose and galactose, which are metabolized by *L. lactis*, could also be used as substrate for mannitol production by using its native metabolism. However, the efficiency of using these sugars for mannitol production are different.

## 5.2 Future directions

### 5.2.1 Finding out the gene responsible for M1Pase activity

In *L. lactis*, M1P could be dephosphorylated to mannitol by an unknown enzyme. This process is suggested to be involved in inducer expulsion, a unique mechanism only found in few Gram-positive bacteria, including *L. lactis*, where intracellular sugar phosphates are dephosphorylated and exported when preferred sugars are present. However, to date, there is no such a characterized phosphatase responsible for this process. To study the mannitol production in *L. lactis*, it is important to find this phosphatase and harness its expression.

### 5.2.2 Optimize the expression of mannitol operon

*L. lactis* mannitol operon is tightly regulated by *ccpA* and *mtlR* together. Although inactivating *ccpA* results in strong derepression of the promoter activity of mannitol operon, it also makes a great change to the cells since *ccpA* is a global transcriptional regulator. Instead of directly inactivating the *ccpA* gene, it is perhaps more optimal to inactivate its binding site in mannitol operon or control the phosphorylation status of Ser46-Hpr. On the other hand, it is suggested that the activity of *mtlR* is affected by its own phosphorylation status. However, MtlR is still not a well-characterized protein in Gram-positive bacteria, and *L. lactis* MtlR was shown to

differ significantly compared with other studied MtlR. Therefore, for optimizing mannitol production in *L. lactis*, it is desirable to have a better understanding on its MtlR.

### 5.2.3 *L. lactis* is a promising host for mannitol production

Mannitol is a six-carbon polyol that could be used in food and therapeutic areas. Because *L. lactis* has been widely used in the food industry, developing mannitol producing *L. lactis* is considered a promising strategy for introducing mannitol to food products, which conforms to the concept of improving health. In this study we have validated that *L. lactis* could produce mannitol from all sugars that were metabolized in glycolysis, which is a great advantage for using *L. lactis* as a mannitol producer. In contrast to heterologous fermentative LAB, which could only produce mannitol from fructose and its derivates, *L. lactis* could utilize a much broad repertoire of sugars as substrate, which could extensively broaden its applications in future food production.

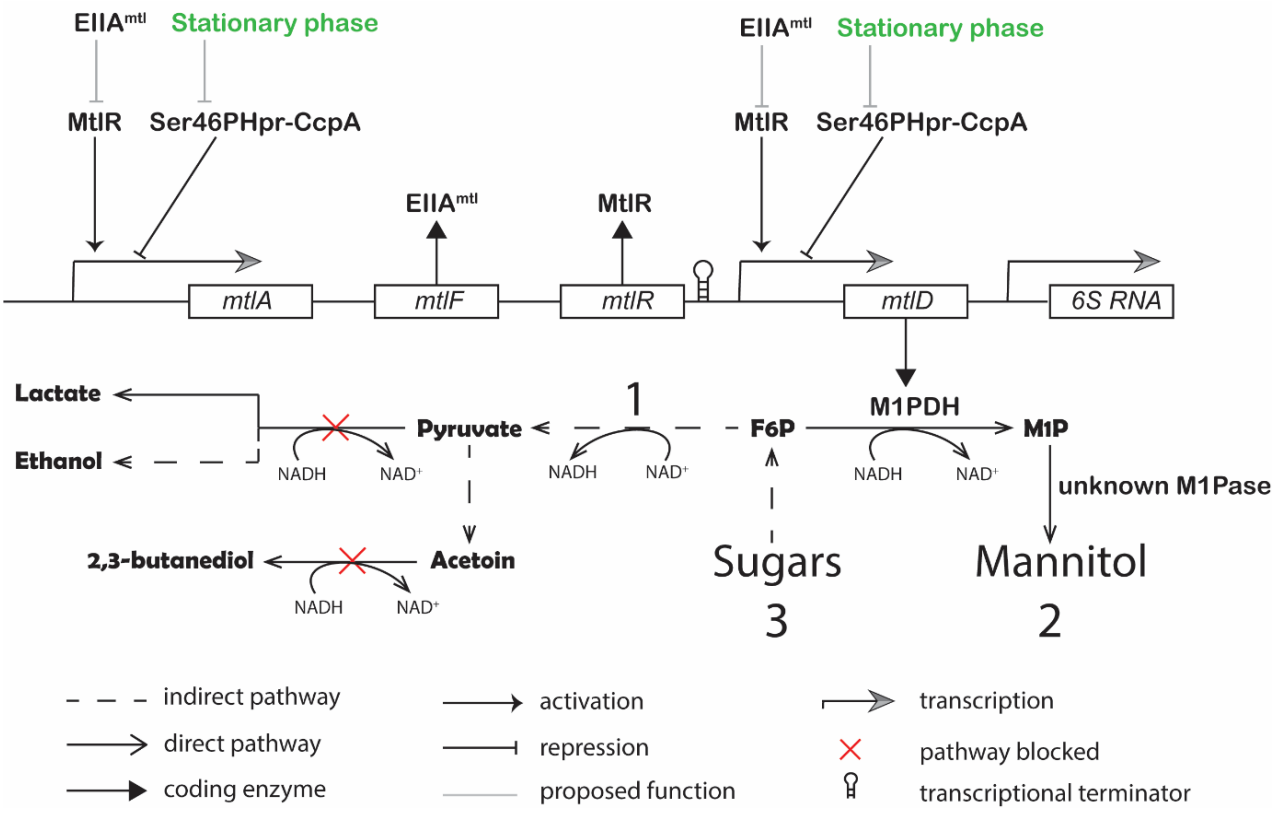


**Figure 5.1. Overview of mannitol production in *L. lactis*.** F6P, Fructose-6-phosphate; M1P, Mannitol-1-phosphate; M1PDH, Mannitol-1-phosphate dehydrogenase; M1Pase, Mannitol-1-phosphatase; Ser46PHpr, Serine 46 phosphorylated Hpr; Numbers indicate the portion.

# Appendix

## I. Bioinformatics for mannitol operon

Bioinformatics analysis for *mtlA* promoter

**Table S1(a). Sequences upstream of the *mtlA* gene**

| Strains | Type | Sequence[a] |
|---|---|---|
| MG1363, CS4099, CS4364, Ace001 | wild type | tggcacctccttttgtgtcaggatttt tatttacagtcttattggtagcggtta caatataattatattagttactatgta aaggagtctgaaatg |
| MG1363-M, CS4099-M, Ace001-M | type I | tggcacctccttttgtgtcaggatttt tatttacagtcttattggtagcggtta **T**aatataattatattagttactatgta aaggagtctgaaatg |
| CS4363-M | type II | tggcacctccttttgtgtcaggatttt tatttacagtcttattggta**T**cggtt acaatataattatattagttactatgt aaaggagtctgaaatg |

[a]Mutation is indicated by shade; Start codon of *mtlA* gene is indicated by underline.

**Table S1(b). Promoter prediction using online tool BDGP**
(http://www.fruitfly.org/seq_tools/promoter.html)

| promoter | | predicted TSS[a] | type I[b] | type II[c] |
|---|---|---|---|---|
| start | end | | | |
| 8 | 53 | 48 | 55 | 48 |
| 24 | 69 | 58 | | |
| 35 | 80 | 75 | | |

[a]TSS: transcriptional start site. [b]Position of SNV for type I mutants. [c]Position of SNV for type II mutants. Numbers are counted from tggcacctcc in Table S1a.

**Table S2. Protein sequence alignment for mtlR between *L.lactis* and other Gram-positive bacteria**

| Bacteria | Length | Identities | Positives | Gaps |
|---|---|---|---|---|
| *L. lactis* | 644 | 100% | 100% | 0 |
| *B.subtilis* | 694 | 26% | 48% | 11% |
| *G.stearothermophilus* | 697 | 25% | 47% | 12% |
| *S.mutans* | 650 | 28% | 51% | 5% |
| *C.acetobutylicum* | 684 | 21% | 44% | 11% |

Notes: Positive, substitution with amino acids with similar chemical properties.

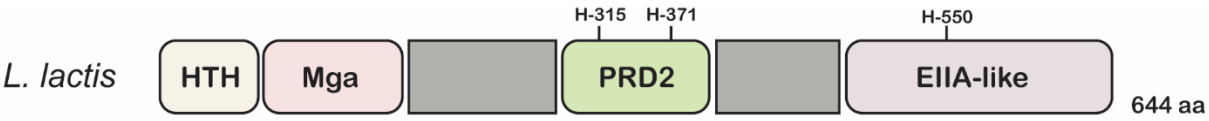


**Figure S1.** Prediction of domains in *L. lactis* mtlR by using Pfam and multiple sequence alignment with *B. subtilis* and *L. casei* mtlR.


**Figure S2.** Transcriptional information from RNA sequencing results for *mtlA* and *mtlD* gene. **a,** Reads mapped in *mtlA* promoter region, sample was derived from exponential Ace001M cells growing in mannitol. **b,** Reads mapped in *mtlD* promoter region, sample was derived from stationary Ace001M cells grown in glucose. The reads visualization is realized by Geneious. *L. lactis* genome (Accession number: NC009004) is used as reference.

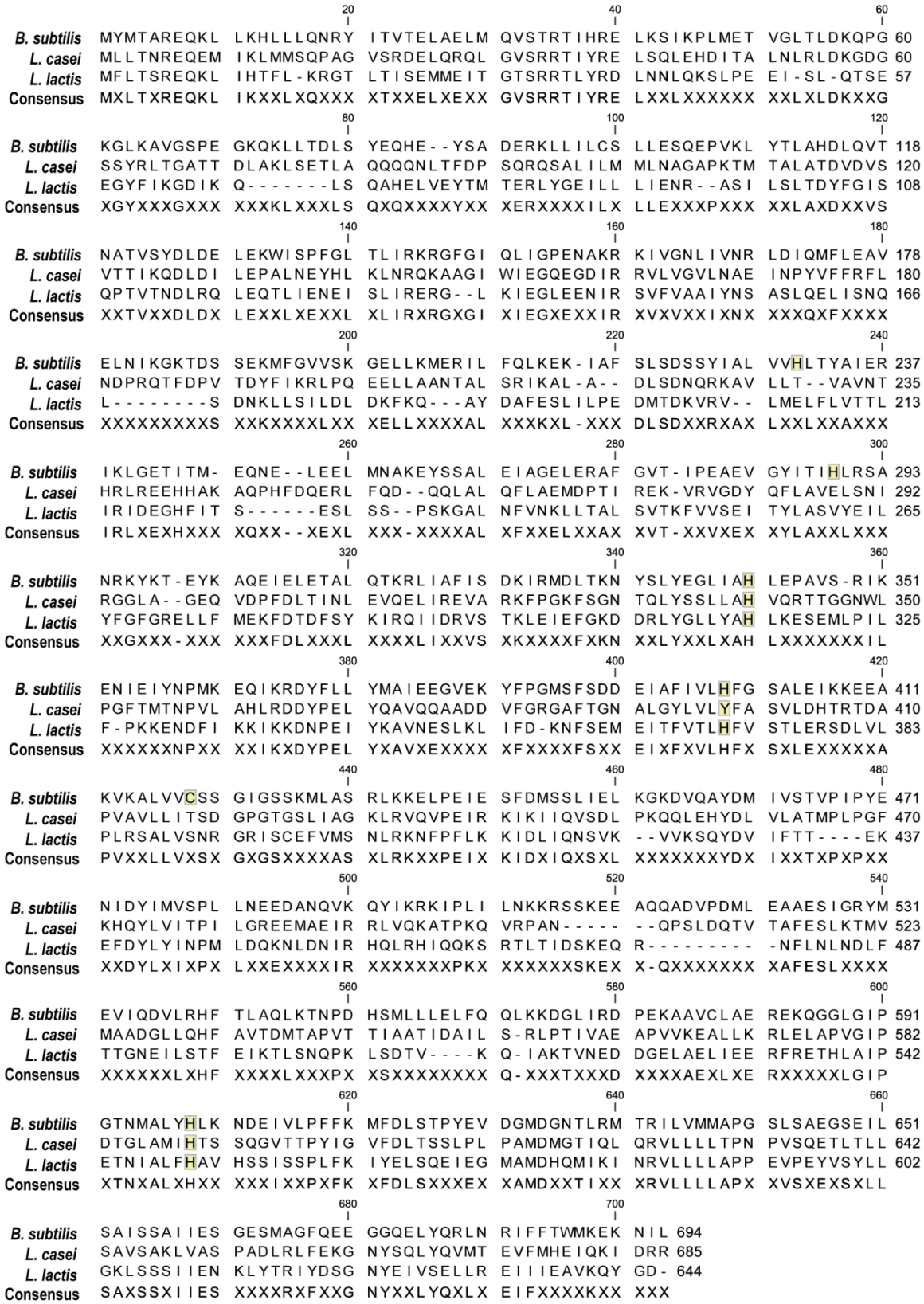


**Figure S3.** Alignment of mtlR amino acids sequence among *B. subtilis, L. casei and L. lactis*. The conserved histidine residues were indicated with shade.

## II. Characterization of growth in different strains

**Table S3. Growth performance of mannitol adapted strains and their parental strains in M17 supplemented with 0.5% mannitol**

| Strains | μ |
|---|---|
| MG1363 | <0.060 |
| MG1363-M | 0.402 |
| CS4099 | <0.060 |
| CS4099-M | 0.285 |
| CS4363 | <0.060 |
| CS4363-M | 0.272 |
| Ace001 | <0.060 |
| Ace001-M | 0.255 |

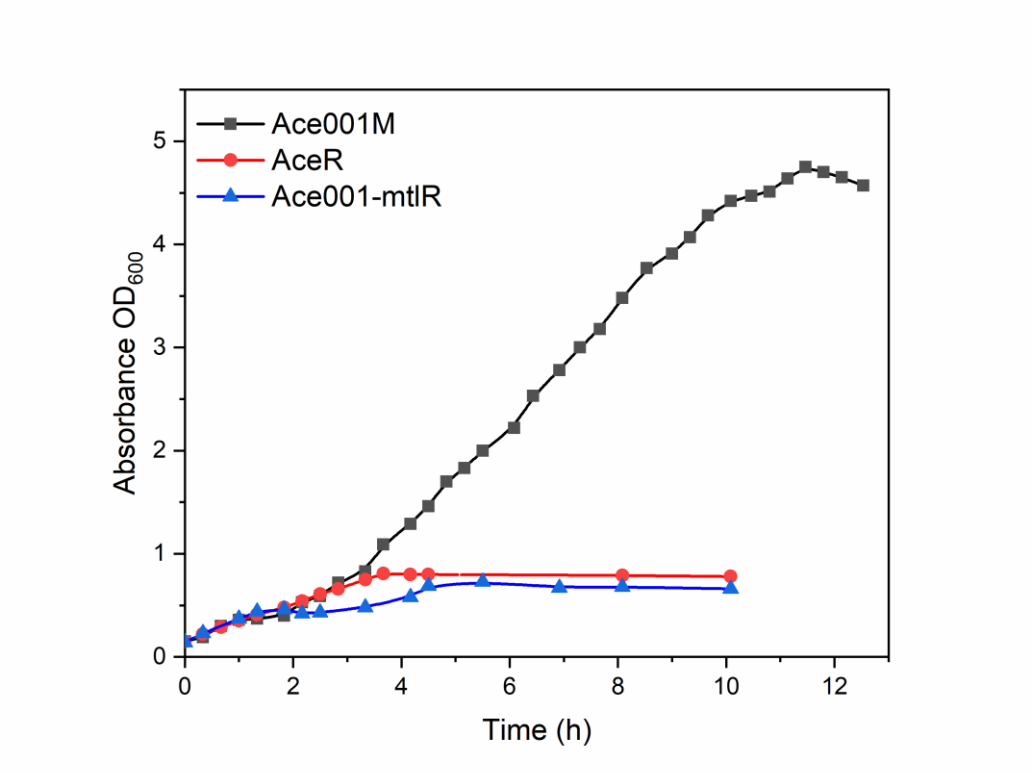


**Figure S4.** Growth curve for different strains growing in 1% mannitol (aerobic). 5 ug/ml tetracycline was used for growing Ace001-mtlR.

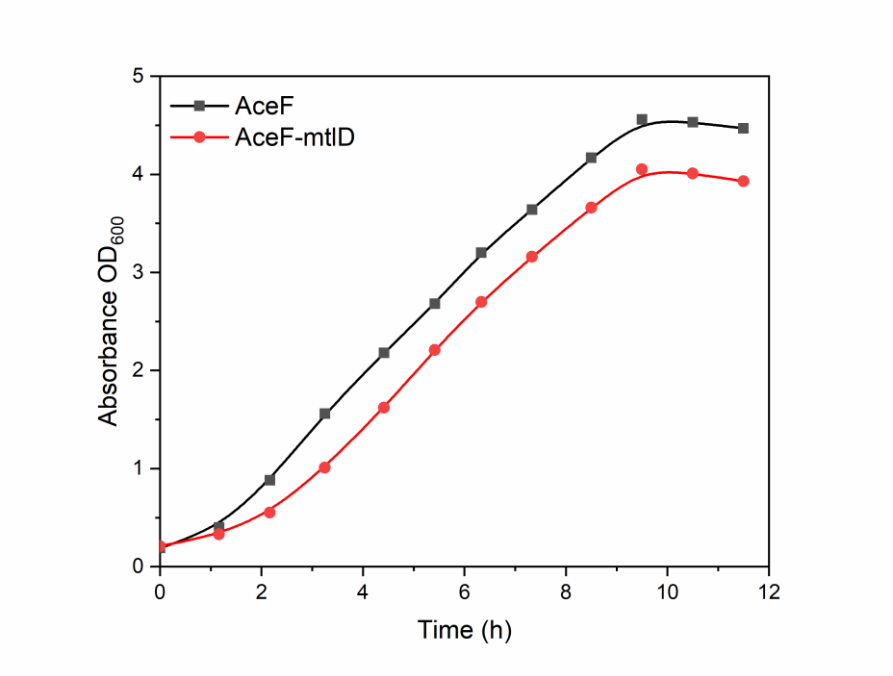


**Figure S5.** Growth curve for AceF and AceF-mtlD growing in 1% glucose (aerobic).

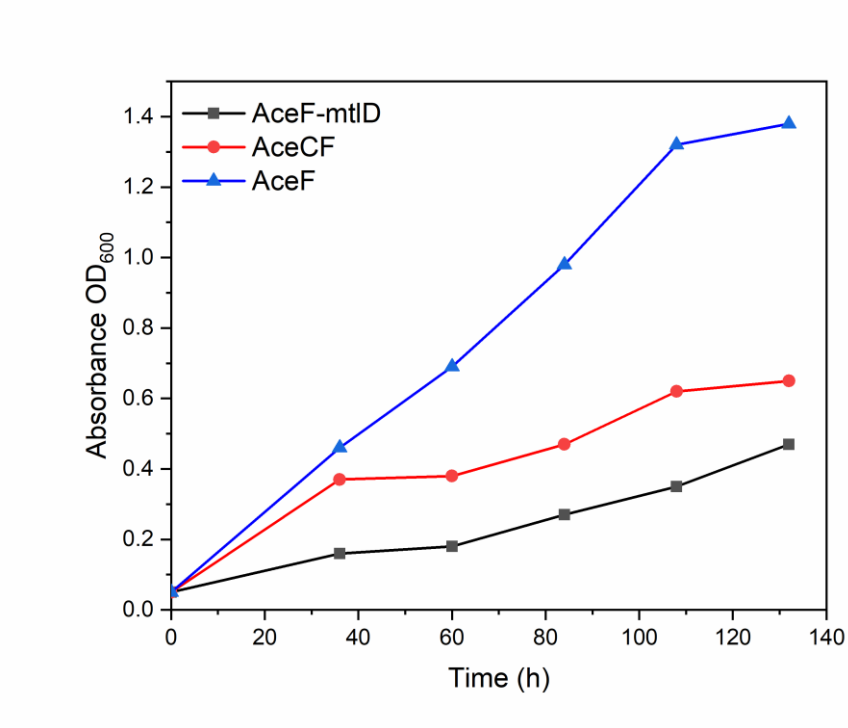


**Figure S6.** Growth curve for different strains growing in 1% glucose (semi-anaerobic). 35mM arginine was added in all treatments. For growing AceF-mtlD, 2 ug/ml tetracycline was added.

**III. Mannitol production in AceF in different conditions**

**Table S4. Data of Figure 4.1a, Yield and titer of mannitol achieved in AceM cultures initiated at a low cell density of $OD_{600}$ = 0.5**

| Treatments | Mannitol titer in particular time point (mM) | | | | Yield |
|---|---|---|---|---|---|
| | 24h | 49h | 72h | 96h | |
| 240rpmAr | 1.45 | 1.54 | 1.62 | 1.62 | 0.02±0.00 |
| 240rpm | 1.78 | 1.70 | 1.81 | 1.81 | 0.02±0.00 |
| static | 1.83 | 2.34 | 2.82 | 3.39 | 0.46±0.13 |
| L100 | 1.83 | 2.63 | 3.19 | 3.60 | 0.53±0.13 |
| H100 | 1.90 | 2.63 | 3.03 | 3.78 | 0.35±0.08 |
| L100Ar | 1.77 | 2.79 | 3.57 | 5.03 | 0.57±0.09 |
| H100Ar | 1.83 | 2.94 | 3.69 | 5.13 | 0.44±0.10 |
| staticAr | 1.63 | 3.13 | 4.63 | 6.18 | 0.58±0.04 |
| L50Ar | 1.95 | 3.50 | 5.11 | 7.28 | 0.29±0.03 |
| L50 | 1.98 | 3.12 | 5.30 | 7.38 | 0.18±0.12 |
| H50Ar | 2.06 | 4.02 | 5.89 | 7.95 | 0.22±0.04 |
| H50 | 1.88 | 3.67 | 6.39 | 10.34 | 0.18±0.03 |
| H25 | 2.79 | 6.33 | 11.17 | 11.65 | 0.14±0.02 |
| L25 | 2.55 | 5.25 | 8.63 | 11.79 | 0.15±0.02 |
| H25Ar | 2.85 | 6.20 | 9.36 | 12.36 | 0.17±0.03 |
| L25Ar | 2.76 | 6.75 | 10.76 | 14.54 | 0.23±0.04 |

H indicates high shaking speed (60 rpm) and L indicates a low shaking speed (20 rpm); 240rpm indicates that the shaking speed used was 240 rpm; Static refers to cultures not being actively aerated, slow diffusion of oxygen possible; Numbers after H/L indicate the volume of medium used in 100 ml flasks; Ar after numbers, indicates addition of 35 mM of arginine. Yields are calculated based on data collected at different time points during the experiment, and shown with standard deviations

**Table S5. Data of Figure.4.1b. Yield and titer of mannitol achieved in AceM cultures initiated at a high cell density of $OD_{600}$=4.5**

| Treatments | Mannitol titer in particular time point (mM) | | | Yield |
|---|---|---|---|---|
| | 17h | 45h | 73h | |
| Static | 4.79 | 7.17 | 8.24 | 0.57±0.01 |
| L75 | 5.42 | 8.04 | 10.28 | 0.49±0.09 |
| L100 | 5.60 | 10.18 | 12.95 | 0.56±0.03 |
| H100 | 5.80 | 11.05 | 13.35 | 0.52±0.00 |
| L50 | 6.31 | 10.09 | 13.53 | 0.41±0.09 |
| H75 | 6.48 | 12.27 | 14.29 | 0.49±0.03 |
| H50 | 7.14 | 12.83 | 15.01 | 0.41±0.03 |
| H50Ar | 9.58 | 22.44 | 27.34 | 0.46±0.02 |
| StaticAr | 8.84 | 20.17 | 27.97 | 0.63±0.02 |
| L50Ar | 10.35 | 21.72 | 28.33 | 0.47±0.04 |
| H75Ar | 10.02 | 23.01 | 29.62 | 0.54±0.03 |
| L75Ar | 9.61 | 21.43 | 31.07 | 0.58±0.00 |
| H100Ar | 9.75 | 23.61 | 32.79 | 0.61±0.01 |
| L100Ar | 9.39 | 22.18 | 33.38 | 0.60±0.00 |

H indicates high shaking speed (60 rpm) and L indicates a low shaking speed (20 rpm); Static refers to cultures not being actively aerated, slow diffusion of oxygen possible; Numbers after H/L indicate the volume of medium used in 100 ml flasks; Ar after numbers, indicates addition of 35 mM of arginine. Yields are calculated based on data collected at different time points during the experiment, and shown with standard deviations.

## IV. Mannitol production in growing-limited condition

### Description

So far, we have relied on the two-stage fermentation setup to produce mannitol. We decided to investigate whether some of the *L. lactis* derivatives mentioned in the previous section were able to grow and produce mannitol under semi-conditions. As shown in Figure S7, all the strains investigated grew slowly, most likely due to inefficient regeneration of $NAD^+$. Compared to AceF, most of other strains gave less than half biomass. We deduced this is because of the plasmid burden and the use of antibiotics in the culture medium, which is similar to the observations from stationary cells. The mannitol titer ranged from 3.3 mM to 11.9 mM whereas the yield of ranging from 28.2% to 43.1%. The mannitol yield increased along with the culturing time, which makes sense because as the dissolved oxygen in the culture medium is gradually consumed as cells grow. Deleting *ccpA* was not beneficial for growth and mannitol production under static conditions, which is similar to what we found for the two-stage fermentation setup. Increasing the M1PDH activity was not beneficial either, which was unexpected as overexpressing M1PDH increased the mannitol titer in the two-stage fermentation setup. Among the cells treated with antibiotics, overexpressing *PTA* resulted in faster growth and higher mannitol titer, suggesting these strains encountered severe ATP shortage during oxygen limited growth. Unlike cells under stationary condition, we observed overexpressing *mtlD* under growing condition would not lead to a big enhancement on mannitol production, as well as growth, which further explained the poor performance on mannitol production detected in AceCF. Furthermore, different from cells incubated in stationary phase, acetoin seems to be the main product besides mannitol, suggesting a great difference in cellular metabolism between growing cells and stationary cells.

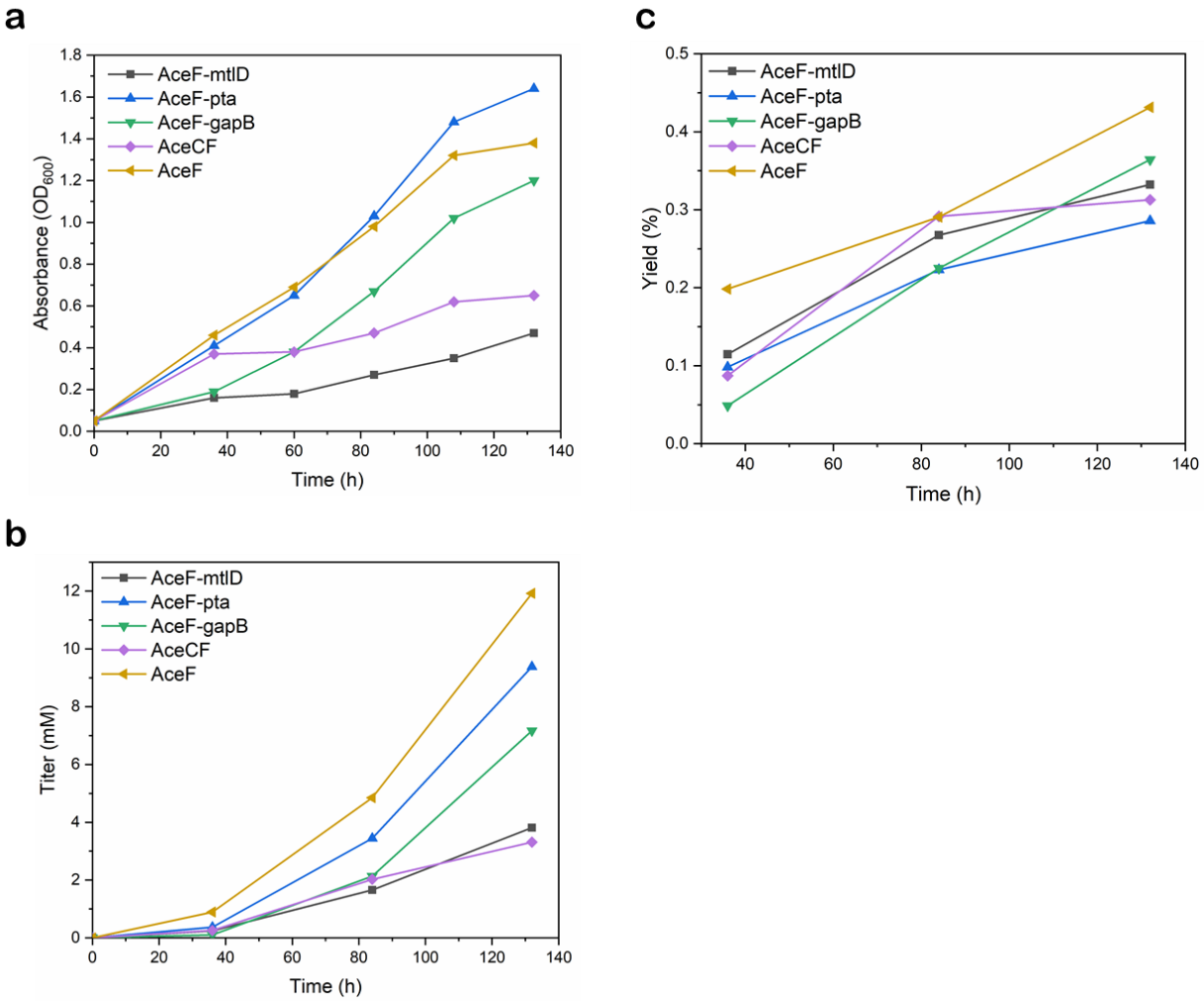


**Figure S7. Mannitol production in growing *L. lactis* with different genetics background.** (a) Growth curve. (b) mannitol titer. (c) mannitol yield. This experiment performed without shaking, only air diffusion.

**Table S6. Mannitol production in *L. lactis* with different genetic background by using slow growing cell (aeration-limited)**

| Strains | Mannitol (mM) | Yield (%) | Acetoin (mM) | Yield (%) | Biomass ($OD_{600}$) |
|---|---|---|---|---|---|
| AceF | 11.9 | 43.1 | 14.9 | 54.0 | 1.38 |
| AceCF | 3.3 | 28.3 | 6.88 | 64.9 | 0.65 |
| AceR-mtlD$^{\uparrow}$ | 3.8 | 33.2 | 7.39 | 64.4 | 0.47 |
| AceF-pta$^{\uparrow}$ | 9.4 | 28.6 | 15.6 | 47.6 | 1.64 |
| AceF-gapB$^{\uparrow}$ | 7.2 | 36.4 | 11.7 | 59.2 | 1.2 |

**Mannitol production by using growing cells**

**Description**

Our results have validated that *L. lactis* is able to produce mannitol from fructose, maltose and galactose by using two-stage fermentation. To test if *L. lactis* could produce mannitol from these sugars under growing condition, AceF and AceCF were inoculated at $OD_{600}$=0.05 in fructose, maltose and galactose under oxygen limited condition. As shown in Figure S8, in this condition, except AceCF from fructose, all cells could slowly grow and produce mannitol but less efficient than those grow in glucose (Figure S7), Interestingly, by deleting *ccpA*, mannitol production from fructose and galactose, but not from maltose were significantly hampered, which were different from the results obtained by using stationary cells (Figure S8).

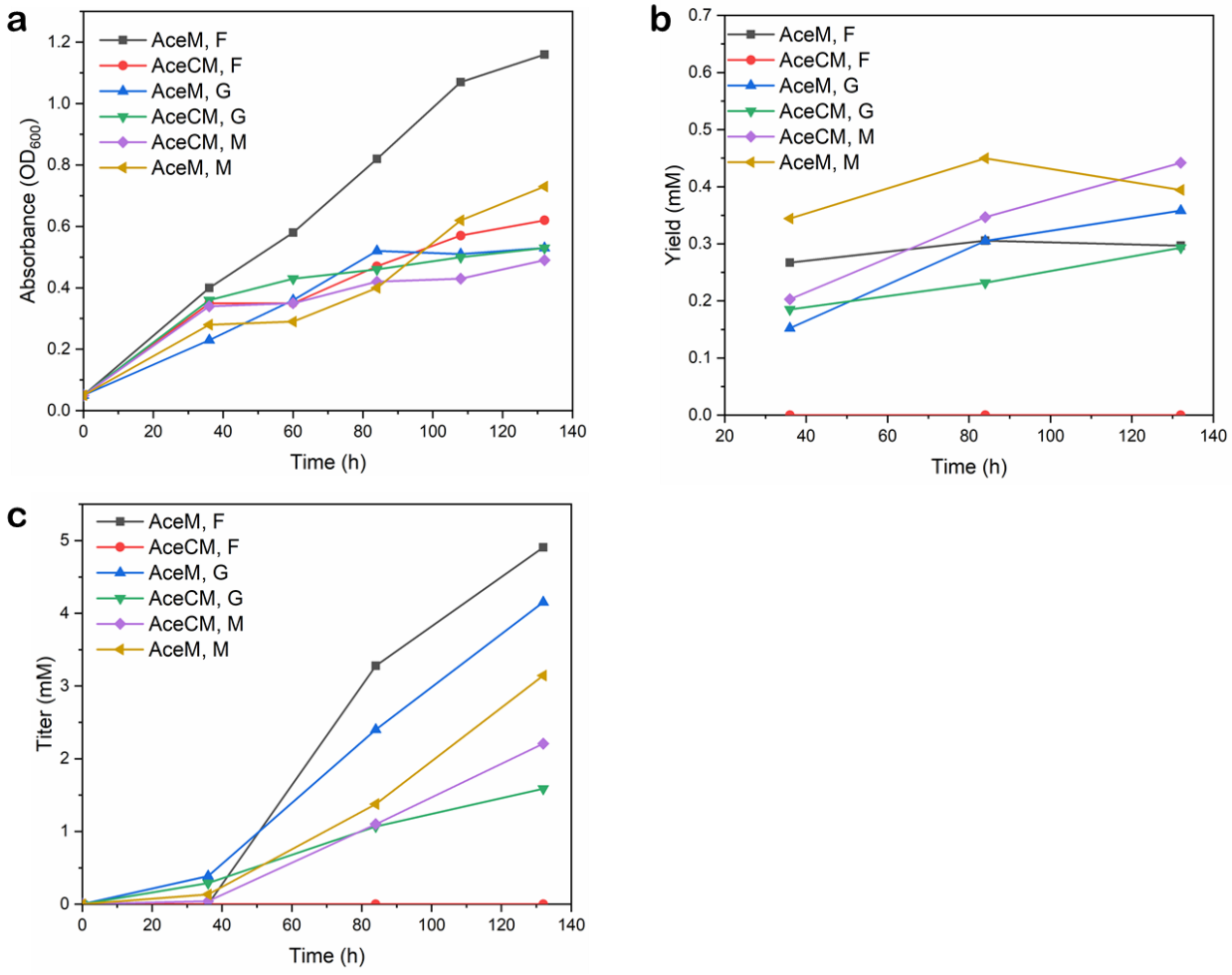


**Figure S8. Mannitol production in growing *L. lactis* from different sugars.** (a) Growth curve. (b) mannitol titer. (c) mannitol yield. This experiment performed without shaking, only air diffusion.

**Table S7. Mannitol production in *L. lactis* from different sugars by using slow growing cell (aeration-limited)**

| Sugars | Strains | Mannitol (mM) | Yield (%) | Acetoin (mM) | Yield (%) | Biomass ($OD_{600}$) |
|---|---|---|---|---|---|---|
| Fructose | AceF | 4.90 | 29.7 | 8,17 | 49.4 | 1.16 |
| Fructose | AceCF | 0 | 0 | 6.77 | 99.2 | 0.62 |
| Maltose | AceF | 3.14 | 39.4 | 3.97 | 49.8 | 0.73 |
| Maltose | AceCF | 2.21 | 44.2 | 2.94 | 58.9 | 0.49 |
| Galactose | AceF | 4.15 | 35.8 | 7.16 | 61.7 | 0.53 |
| Galactose | AceCF | 1.59 | 29.3 | 3.53 | 65.2 | 0.53 |