Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in
*Lactococcus lactis* by metabolic engineering☆

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Abstract

Lactic acid bacteria display a relatively simple metabolism wherein the sugar is converted mainly to lactic acid. The extensive knowledge of metabolic pathways and the increasing information of the genes involved allows for the rerouting of natural metabolic pathways by genetic and physiological engineering. We discuss several examples of metabolic engineering of *Lactococcus lactis* for the production of important compounds, including diacetyl, alanine and exopolysaccharides. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

Lactic acid bacteria (LAB) are used world-wide in the industrial manufacture of fermented food products. Their most important application in this respect is undoubtedly in the dairy industry, where these micro-organisms are used to convert milk or milk-derived products to an enormous variety of fermented dairy products. However, next to milk also other unprocessed food materials, like meat and vegetables, are subjected to fermentation by LAB on an industrial scale. In milk these bacteria encounter lactose as the major carbon source, but they have the capacity to use a number of other mono- and disaccharide substrates. The major product of these fermentations is lactic acid, which plays a crucial role in protection of the final fermented product against spoilage. Besides this acidification that acts as a natural preservative effect, LAB metabolism is essential for development of desired product properties like flavor, shelf-life, and texture. The metabolic conversions that generate these end-products vary widely, depending on the lactic acid bacterium. Metabolic engineering strategies that involve inactivation of undesired genes and/or overexpression of existing or novel ones have been used to create rerouting of the metabolic fluxes by changing the energy metabolism or the concentrations of metabolic intermediates or of existing or completely new end-products. To generate a rational approach for these strategies, it is essential to understand the pathways that are manipulated, their fluxes, control factors, and the genes involved. Knowledge of the traditional pathways recognized in LAB and the characterization of new ones, in combination with physiological studies, has led to an extensive insight in LAB metabolism. Proper appreciation of the application potential of LAB as cell factories is related to several features that are shared by these micro-organisms. In general they have a long history of safe use and grow rapidly on media that are derived from milk and contain lactose as the major carbon source, which is converted to a number of interesting metabolic end-products during fermentation. Moreover, LAB are applied world-wide as starter cultures in industrial food fermentations, implying that LAB with modified metabolic pathways cannot only be used in bioreactors but also for in situ production of flavor, texture, or health promoting compounds in food products or even in the gastro-intestinal tract of consumers of food. Several other features of LAB, like

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their relatively simple metabolism, limited biosynthetic capacity, and apparent lack of gene multiplicity, illustrate their advantages for metabolic engineering purposes compared to other microorganisms like yeast or fungi.

We will review several strategies that have been used to reach (efficient) rerouting of carbon metabolism in LAB. The focus will be on the best studied LAB, *Lactococcus lactis*. We will highlight some important genetic tools as well as their application in some recently achieved examples of efficient rerouting of carbon metabolism. In addition, several examples will be given of metabolic engineering strategies that are currently investigated and could result in efficient cell factory systems in the near future.

2. Genetic tools for LAB

Over the past decades, advances in the genetics of LAB have resulted in the development of a large variety of genetic techniques, including transformation procedures, integration, vector, and gene expression systems [1]. A large number of genes and operons from LAB have been characterized and functionally analyzed. In addition, the determination of the entire genome sequence of *L. lactis* was recently reported to be completed [2], whereas genome sequencing of several other LAB, including those belonging to the genera *Lactobacillus* and *Streptococcus* has been initiated. Moreover, several sustainable and safe selection systems have been developed that allow application in the food industry and therefore are designated “food-grade” [3]. These developments generate sophisticated tools and a mass of genetic information that can be used by LAB as a cell factory.

Several recent reviews give an excellent overview of both controlled and constitutive gene expression systems that are available for *L. lactis* and other LAB [1, 4–6]. Therefore, the development and characteristics of individual gene expression systems will not be discussed here. Nevertheless, two recently developed systems seem worth mentioning because of their proven applicability as tools for genetic engineering to facilitate efficient rerouting of metabolic fluxes. The first system was developed by the construction and screening of synthetic promoters that were based on the *L. lactis* consensus promoter elements (i.e. −35 and −10 hexamers; [1], but in which the intervening spacer region had been randomized [7]. This approach resulted in a large set of constitutive promoters that display a wide range of expression efficiencies [7]. It seems likely that these promoters (or a subset of these promoters) might also be functional in other LAB and could thus provide a general system for constitutive gene expression in these bacteria, which is a very useful characteristic in certain metabolic engineering strategies where gene expression is preferentially maintained at a stable level. Although these constitutive promoters hold great promise, no specific example of their application has been reported so far, which is probably due to their recent development. The second system is also of specific interest because it allows controlled gene expression in a wide variety of lactic acid and other Gram-positive bacteria [8,9]. This system has been designated the gisin controlled expression (NICE) system as it is based on the autoregulatory characteristics of the antimicrobial peptide gisin that is produced by several strains of *L. lactis* (Fig. 1). Gisin acts as extracellular peptide pheromone that is involved in activation of its own biosynthesis [10,11]. Signal transduction occurs via a typical bacterial two component signal transduction machinery, consisting of a membrane anchored sensor protein (NisK) and a cytoplasmic response regulator protein (NisR). Upon interaction with gisin, NisK is likely to autophosphorylate, followed by phosphotransfer to NisR, thereby generating the activated form of the latter component that activates transcription of the target genes (Fig. 1). Two gisin-responsive promoters have been identified, preceding the *nisA* and *nisF* genes [12], and coupling of such a promoter to a gene of interest allows activation of expression of that gene by the addition of the food-grade inducer gisin to the growth medium, or in gisin containing milk products. A linear dose-response re-
A relationship between the amount of inducer (nisin) and the level of nisA promoter-driven gene expression allows modulation of gene expression in a dynamic range of approximately 1000-fold. Moreover, due to the nisA promoter silence in the absence of nisin, this system allows the controlled expression of lethal genes [13,14]. By introduction of the signal transduction system encoding genes, nisRK, in other lactic acid bacteria, the NICE system was functionally implemented in *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* [8,13]. This system has been used for the efficient expression of homologous and heterologous enzymes in *L. lactis* and has proven to be very useful for metabolic engineering purposes (see below).

### 3. Sugar transport and glycolysis in *L. lactis*

*L. lactis* can use a variety of mono- and di-saccharides as carbon source, including glucose, fructose, galactose, lactose, maltose, mannose, and cellobiose. Three different systems for sugar import are known in *L. lactis*. Primary transport systems display a direct coupling of sugar import to ATP hydrolysis via a transport specific ATPase [15]. In secondary sugar transport systems the import of sugar is coupled to transport of ions or other solutes [16]. Finally, sugars that are imported via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) are phosphorylated during transport. This system transfers the phosphate group from PEP, resulting in the formation of pyruvate, to the incoming sugar via two cytoplasmic phosphocarrier proteins: enzyme I and HPr [17]. In lactic acid bacteria, many sugars are imported via this PTS system, which is an energetically efficient system for sugar transport because sugar import is directly coupled to sugar phosphorylation at the expense of one ATP that would otherwise be generated by PEP conversion to pyruvate by pyruvate kinase (pyk) (Fig. 2). Recently, the *ptsHI* operon of *L. lactis*, encoding the general PTS components HPr and enzyme I, respectively, has been cloned and characterized. HPr plays an important role regulation of sugar utilization, both at the level of transcription of genes involved in use of a specific sugar and at the level of sugar transport. This phosphocarrier protein contains two residues that are target for phosphorylation. A histidine residue in the N-terminal part of the protein, that is a target for enzyme I mediated phosphorylation and the resulting HPr-(His-P) primarily plays a role in PTS sugar transport, but can also influence the use of non-PTS sugars [18,19]. The second HPr-residue that is a target of phosphorylation is a conserved serine residue in the C-terminal part of HPr. The HPr-(Ser-P), like HPr-(His-P), plays a complex role in sugar use, where it can, (i) allosterically control PTS and non-PTS permease activity, thereby regulating sugar import, (ii) control the activity of a sugar-phosphate phosphatase, thereby playing a role in the phenomenon called inducer exclusion and (iii) interact with the catabolite control protein CcpA that negatively controls the expression of a large number of genes or operons that are subject to catabolite repression [18]. Moreover, it has recently been shown that the lactococcal CcpA, besides its role in catabolite repression, also regulates sugar metabolism by affecting the glycolytic flux through activation of the *las* operon [20].

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**Fig. 2.** Schematic representation of the lactose utilising pathway used by *L. lactis*, leading to pyruvate. Known enzymes are indicated using abbreviations based on established genetic nomenclature. Triose-P represents both glyceraldehyde-3P and dihydroxyacetone-P, from which the latter is converted to glyceraldehyde-3P as indicated. Links to other figures in this article are indicated.
In dairy fermentations lactose is the main carbon source encountered by *L. lactis* and the pathway used to convert this sugar is relatively well understood (Fig. 2). In many lactococcal strains, the genes involved in lactose use (*lac* operon) are plasmid encoded, and the best described is the lactose mini-plasmid pMG820 [21,22]. This plasmid contains genes involved in lactose-PTS transport (*lacEF*), lactose-6P hydrolysis (*lacG*), and the tagatose-6P pathway (*lacABCD*) (Fig. 2). Expression of the *lac*-operon genes is controlled by the transcriptional repressor encoded by *lacR* and repression of the *lac* gene expression can be relieved by the inducer tagatose-6P, which is an intermediate of the tagatose-6P pathway (*lacABCD*) (Fig. 2). An elegant example of engineering of lactose utilisation in *L. lactis* was described in 1985 by Thompson et al. By classical mutagenesis, a *L. lactis* double mutant was constructed that lacked both the glucokinase (*glk*; Fig. 2) activity and the mannose PTS system involved in glucose import. As a result, when this strain was grown on lactose, the galactose-6P moiety of this disaccharide can be converted via the tagatose-6P pathway, whereas the glucose moiety remains unphosphorylated and is secreted. The lack of a mannose PTS in these cells prevents the subsequent uptake of the expelled glucose. The overall result is an apparent quantitative conversion of lactose in glucose metabolism in lactose grown *L. lactis* can be completely separated [24].

Many of the genes encoding glycolytic enzymes in *L. lactis* have been cloned and characterized. Moreover, a predictive metabolic flux model of lactococcal glycolysis was recently developed on basis of in vivo NMR measurements, allowing qualitative prediction of metabolic shifts upon changes in the environmental conditions [25]. Interestingly, the transcription of the *las* operon [26], encoding three key-enzymes involved in glycolysis and production of lactate, phosphofructo-kinase (*pfk*), pyruvate-kinase (*pyk*) and lactate dehydrogenase (*ldh*) (Fig. 2), has recently been shown to be activated by CcpA [20]. By inactivation of the lactococcal *ccpA* gene, strains were generated that displayed a strongly reduced expression level of the *las* operon genes, which resulted in a shift from homo-lactate to mixed acid fermentation [20]. This study is an elegant example of the possibility of metabolic engineering by manipulation at the level of global regulation, and the availability of the key-regulatory components that exert this control in *L. lactis* (HPr, CcpA and Enzyme I) [19,20] will probably allow further fine-tuning of metabolic engineering strategies in the future.

### 4. Engineering of the pyruvate metabolism

Pyruvate is the key metabolic intermediate; it is the endproduct of the substrate level phosphorylation reactions of the glycolytic pathway and is also produced through the phosphorylation cascade involved in PTS-mediated sugar import (Fig. 2). Pyruvate can be converted to various end-products by *L. lactis*, including acetate, ethanol, diacetyl, acetoin, and 2,3-butanediol (Fig. 3). However, the main endproduct is evidently lactate, which is formed by the lactate dehydrogenase reaction. This reaction acts as main electron sink under anaerobic conditions, leading to homo-lactic fermentation. However, a shift toward mixed acid fermentation has been observed under certain fermentation
conditions, such as carbohydrate limitation [27], diminished rates of sugar metabolism [28], and aerobic conditions [29]. Several genetic engineering strategies have been used to create mutant lactococcal strains that display a significant shift in their metabolism and that produce either one of the endogenous endproducts more efficiently or produce an endproduct that is normally not produced by \( L. \) lactis. Here we will describe examples of efficient rerouting of pyruvate metabolism toward the endproducts diacetyl and alanine.

5. Engineering diacetyl production

One of the endproducts that can be generated from pyruvate is diacetyl (Fig. 3), which is an important flavor compound in dairy products generating the typical butter-aroma. This compound is naturally produced by \( L. \) lactis, especially by \( L. \) lactis subsp. \( lactis \) biovar. \( diacetylactis \), from citrate in co-metabolic fermentation with lactose [30]. During this fermentation the intermediate \( \alpha \)-acetolactate is accumulated, which is chemically (nonenzymatically) converted to diacetyl through an oxidative decarboxylation reaction (Fig. 3). Based on its aroma value, efficient diacetyl (or its precursor \( \alpha \)-acetolactate) production from lactose rather than from citrate by \( L. \) lactis has been one of the targets of various metabolic engineering strategies.

An obvious target for engineering is the lactate dehydrogenase (LDH), and an LDH deficient strain was created by \( ldh \) gene disruption. The mutant displayed a mixed acid fermentation under anaerobic conditions, producing high amounts of formate and ethanol. In contrast, upon aeration a dramatic shift in pyruvate metabolism was observed, leading to increased acetate production and acetoin as the main endproduct [31]. An alternative approach is to increase the amount of enzyme involved in conversion of pyruvate to \( \alpha \)-acetolactate. Two enzymes are known in \( L. \) lactis that catalyse this reaction: \( \alpha \)-acetolactate synthase (ALS; encoded by the \( als \) gene; Fig. 3) [32] and acetohydroxy acid synthase (ILVBN; encoded by the \( ilvBN \) gene; Fig. 3) [33]. Although the \( als \) gene product acts as the catabolic synthase, the \( ilvBN \) gene product is an anabolic synthase that is involved in branched chain amino acid biosynthesis. Overexpression of the \( ilvBN \) genes in \( L. \) lactis grown under aerobic conditions resulted in a 3- to 4-fold increased flux toward acetoin [34], suggesting that the ILVBN activity level was probably low and indicating that this approach could possibly be improved. A 100-fold increase in ALS enzyme level was achieved by cloning the \( als \) gene on a multi-copy plasmid, resulting in a 40% rerouting efficiency of the pyruvate pool towards acetoin under aerobic conditions [31]. Moreover, overexpression of ALS in a LDH-deficient background led to the production of very high amounts of acetoin (and relatively low amounts of \( \alpha \)-acetolactate), accounting for 85% of the pyruvate converted. The approaches described here have been more or less successful in generating lactococcal strains that effectively produce acetoin from lactose or glucose, but have failed to create strains that produce diacetyl efficiently. This is due to the endogenous \( \alpha \)-acetolactate decarboxylase (ALDB; encoded by the \( aldB \) gene; Fig. 3) activity that efficiently converts \( \alpha \)-acetolactate to acetoin. Inactivation of the \( aldB \) gene resulted in strains that produce low levels of diacetyl under aerobic conditions, and the diacetyl production level could be increased by overexpression of the \( ilvBN \) genes in this strain [35]. However, only low absolute levels of diacetyl production could be achieved, and these cells still produced relatively high levels of acetoin, which is due to the acetoin diacetyl reductase activity (ADR; Fig. 3). Interestingly, an elegant approach to obtain ALDB deficient natural \( L. \) lactis variants has recently been described [36]. Taken together, these results suggest that a strain in which all three mutations are combined (LDH- and ALDB-deficiency and ALS or ILVBN overproduction) could be an effective diacetyl producer. However, presently no such \( L. \) lactis strains have been described, which is possibly explained by putative lethality or poor viability of lactococcal cells that are deficient in both LDH and ALDB.

6. Efficient diacetyl production by cofactor engineering

The intracellular redox balance, most importantly reflected by the NADH/NAD\(^+\) ratio, plays a predominant role in control of the fermentation pattern displayed by \( L. \) lactis [29,37]. Pyruvate reduction by LDH effectively regenerates NAD\(^+\) and thereby effectively transfers reduction equivalents formed during glycolysis to the metabolic endproduct. Interestingly, and illustrative for the energetically unfavorable pathways that will be used by these cells to maintain their redox balance, it has recently been shown that the rate of sugar fermentation by LDH-deficient \( L. \) lactis strains can be increased by co-metabolisation of acetate. The acetate consumed under these conditions is exclusively converted into ethanol at the expense of NADH and ATP, thereby creating a redox rescue pathway for these cells that lack an effective reductive pathway [38]. The stringent redox balance control of fermentation patterns has recently been exploited in a metabolic engineering approach designated cofactor engineering [39]. \( L. \) lactis strains were created in which the redox balance could directly be controlled, which was achieved by exploiting the NICE system for controlled expression of the \( S. \) mutans nox gene. Overexpression in a dynamic range of this water-forming NADH-oxidase (NOX) encoding gene in combination with specific aeriation levels allowed effective control of the NADH/NAD\(^+\) balance [39]. These induced variations in NOX activity resulted in inhibition of all NADH-dependent pathways and a rerouting of the pyruvate pool toward oxidative or NADH-independent pathways. In effect, the pyruvate metabolism was shifted from homolactic toward mixed acid fermentation, and at maximal NOX-induction level combined with boosting of NOX activity by addition of FAD to the growth
medium, an almost complete replacement of lactate by acetate and acetoin (and some diacetyl) could be achieved [39].

Furthermore, by overproduction of NOX in an ALDB-deficient lactococcal background, the pyruvate metabolism could be efficiently rerouted toward \( \alpha \)-acetolactate and diacetyl. These cells appeared to convert more than 80% of the available pyruvate pool to this butter aroma compound and its precursor [40]. Regarding the central control role of the redox balance on lactococcal metabolism, this approach of cofactor engineering in combination with inactivation or overexpression of other enzymatic steps will probably have a strong impact on future metabolic engineering strategies that aim at rerouting toward metabolic endproducts other than lactate. Moreover, cofactor engineering could readily be applied in other lactic acid bacteria because the expression system used (NICE) can be functionally transferred to other bacteria by an easy to handle two-plasmid system [8].

7. Homo-L-alanine fermentation by modified \( L. \) lactis

Most metabolic engineering strategies that have been applied in \( L. \) lactis were aimed at increasing the production of one of the natural lactococcal metabolic endproducts. As an alternative, introduction of a heterologous pathway in \( L. \) lactis could lead to lactococcal variants that produce interesting new products from lactose (or another carbon-source). An example of this type of approach is the over-expression of the \( Zymomonas \) mobilis genes encoding pyruvate decarboxylase (\( pdc \)) and alcohol dehydrogenase (\( adh \)). Expression of this ethanol forming pathway in LDH-deficient \( L. \) lactis cells led to a significant amount of ethanol production (2%) (Hugenholtz, unpublished results). Recently, extremely efficient rerouting of the pyruvate flux was achieved by overexpression of L-alanine dehydrogenase (ALAD), encoded by the \( alaD \) gene derived from \( Bacillus \) sphearcus (Fig. 3) [41]. The \( alaD \) gene was cloned under control of the \( nisA \) promoter and the encoded enzyme accounted for approximately 30–40% of total soluble proteins under maximally induced conditions. In wild-type lactococcal cells this already resulted in a 30–40% rerouting efficiency from lactate toward alanine when resting cells were used as glucose-utilizing biocatalysts in the presence of an excess of ammonium, which is required for the pyruvate to alanine conversion by ALAD. Moreover, introduction of this system in LDH-deficient lactococcal cells and incubation of the resulting cells under the appropriate conditions, resulted in a complete conversion of the pyruvate pool into alanine. The alanine produced consisted of a mixture of both stereo-isomers (\( \text{D-} \) and \( \text{L-} \)alanine), and in all cases D-alanine amounted to approximately 10–15% of the total alanine produced. This partial racemisation of the L-alanine produced is due to the alanine racemase (ALR) activity encoded by the \( alr \) gene of \( L. \) lactis. In order to create a stereospecific L-alanine producing lactococcal variant, the \( alr \) gene was disrupted in the LDH-deficient strain and it could be shown that the resulting strain indeed produced stereospecific L-alanine as the sole endproduct of its fermentation [41]. In conclusion, this strategy of metabolic engineering has led to a complete conversion of \( L. \) lactis from the naturally occurring homo-L-lactate fermentation to a homo-L-alanine fermentation. Similar as described for the redox engineering strategy, the alanine producing capacity can readily be transferred to other LAB using the two-plasmid NICE-transfer system [8]. This extremely successful engineering strategy illustrates the potential of simple rerouting pathways in creating LAB that produce large amounts of completely new compounds. In this case the compound produced, L-alanine, is used as a food-sweetener and in pharmaceutical applications, where it is added in combination with other L-amino acids to standard infusions in preoperative and postoperative nutrition therapy [42].

The system using resting lactococcal cells as biocatalysts for the quantitative conversion of a cheap carbon source (i.e. sucrose, whey, starch) into L-alanine clearly has potential as a new industrial process for the production of this compound as a fine chemical. Another interesting application of alanine producing lactic acid bacteria could be the in situ production of this sweetener in fermented dairy products like buttermilk, yogurt, or cheese. The potential of this latter application of the described system is currently being evaluated by several European laboratories, in a concerted research program.

8. Complex pathway engineering

A major challenge in current and future engineering approaches is the modulation of complex pathways, leading toward interesting endproducts. A good example of such a product is given by the polysaccharides that are naturally produced by some so-called ropy strains of LAB. Recent years have shown increasing interest in the production of exopolysaccharides (EPS) by LAB, caused by their relevance in dairy-product properties like texture and mouthfeel. Based on the food-grade status of LAB, these EPSs could readily be applied as food additives, where they can contribute to several product properties (i.e. viscosity, emulsion-stability). Moreover, it has been suggested that they can be active as prebiotic [43], cholesterol lowering neutra-ceutical [44] or immunomodulant [45,46]. Over the last years, the chemical structure, the molecular biology and genetics of the biosynthesis of various repetitive heteropolysaccharide EPSs produced by several LAB have been studied. In EPS producing \( L. \) lactis, the EPS specific \( eps \) genes are encoded on large plasmids (>20 kb) that can be transferred by conjugation, which concomitantly results in transfer of EPS production [47,48]. \( L. \) lactis strain NIZO B40 produces a phosphopyrulosaccharide with a known structure (Fig. 4B; \( \rightarrow 4\))\([\alpha-1-Rhap-(1\rightarrow2)\][\alpha-\text{d-Galp-1-PO}_4-3\text{-}][\beta-\text{d-Galp-(1\rightarrow4)-d-Glc}-(1\rightarrow4)\text{-}d-Glc}-(1\rightarrow4)]\). The
12 kb NIZO B40 eps gene cluster contains 14 coordinately expressed genes, epsRXABCDEFGHILJKL (Fig. 4A), and is localized on a 40 kb plasmid [47]. Recently, in vitro experiments have shown that the biosynthesis of B40-polysaccharide backbone is initiated by the linkage of a glucose from UDP-glucose to the lipid carrier by priming-glucosyltransferase EpsD [47,50]. Subsequently, the addition of a second glucose from UDP-glucose to the lipid-linked glucose involves the combined activity of EpsE and EpsF, and finally, the addition of the third backbone-sugar moiety, galactose, from UDP-galactose to this carrier bound cellobiose involves the activity of EpsG (Fig. 4B) [50]. So far, the rhamnosyl- and phospho-galactosyl-transferase activities involved in coupling of the side-chain sugar moieties have not been experimentally established but have been proposed to be encoded by epsH and epsJ (Fig. 4B) [50]. Increasing knowledge on EPS structures, combined with the availability of individual biosynthetic components from eps gene clusters could allow directed EPS-engineering approaches aiming at changing its chemical structure (sugar-composition and/or sugar-linkages) or other relevant features like its chain-length. An essential first step in this direction has been realized by the demonstration that a B40-EpsD deletion mutant could functionally be complemented, not only by the homologous EpsD gene itself, but also by heterologous priming glucosyltransferase encoding genes [51]. Recently, other examples that hint at the possibilities for production of heterologous or new EPS structures in L. lactis have been reported. Expression of the genes involved in type 3 capsular polysaccharide (CPUs) production in S. pneumoniae resulted in high level production of the type 3 polysaccharide by L. lactis [52]. The entire S. thermophilus Sf6 eps gene cluster was expressed in L. lactis, resulting in production of low but significant amounts of EPS of a different structure as compared with the EPS produced by the original S. thermophilus strain [53]. These different approaches offer good perspective for future eps engineering in L. lactis (or in other LAB), and show that this host could be used for the expression of oligo- or polysaccharides that have certain desirable characteristics. Besides the specific eps genes, EPS production also requires a number of so-called housekeeping genes that are
involved in the biosynthesis of the EPS building blocks, the nucleotide-sugars. Possibly, the relatively low EPS production levels displayed by LAB could be increased by increasing the metabolic flux toward these nucleotide-sugars (Fig. 4B). Therefore, the physiology of nucleotide sugar biosynthesis in relation to EPS production levels should be evaluated to eventually use metabolic engineering as a means to increase EPS production by LAB. Currently, the role of the genes that encode potentially important enzymes in these metabolic pathways are being investigated (i.e., galE, galU, and rfbABCD; Fig. 4B). Moreover, this metabolic engineering approach will be supported by the simultaneous development of a kinetic flux model that describes the contribution of the individual enzymes to the level of the various nucleotide-sugars generated. Taken together, these strategies could eventually generate a rational for improvement of the production levels of a complex endproduct like EPS, which is crucial when these EPS producing LAB are to be used as food-additive producer organisms. In contrast, the requirements for application of modified LAB that produce a certain desirable oligo- or polysaccharide in situ are determined more by their functionality (e.g. role as neutraceutical; probiotic, or vaccine, generating texture or mouthfeel), than by their production level. It is expected that the engineering of EPS and its biosynthesis pathway in LAB will develop into an important objective in future metabolic engineering research, especially since the in situ production of certain complex polysaccharides that generate a health benefit to the fermented products seems a very attractive application.

9. Conclusion

The examples described above illustrate the potential of lactic acid bacteria as cell factories to produce desirable products during fermentation. It is clear that the metabolic flexibility of these bacteria is larger than could be expected on basis of the simple fermentation pattern they display naturally. One possible application of engineered LAB is their use as real cell factories that displays an efficient conversion of the carbon source into a certain desirable endproduct that is subsequently separated from the producing cells and used as additive in food or other applications. The other, and maybe more important application of engineered LAB as producers of certain products, is their potential for in situ production (engineered starter cultures) of a compound that contributes to the functional characteristics (e.g. taste, texture, nutritional value, health benefits) of the fermented product.

We have described a few examples of metabolic engineering strategies used in LAB. Future engineering of LAB requires the identification of new high-value endproducts that can be targeted by engineering strategies leading to, for example, the tailor-made oligo and polysaccharides mentioned, or to compounds like vitamins, antioxidants, or compatible solutes. In most cases these compounds are generated by a complex biosynthetic pathway, the engineering of which will be an important challenge for future metabolic research in LAB.

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