Metabolic Engineering

of Exopolysaccharide Production in *Lactococcus lactis*

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Abstract

Abstract

Exopolysaccharides (EPS) produced by lactic acid bacteria are important structural components in fermented foods. In addition, they may confer health benefits to the consumer, as mouse model studies have indicated that EPS may have immunostimulatory, anti-tumoral, or cholesterol-lowering activity. *Lactococcus lactis* strain NIZO B40 produces phosphorylated EPS with a branched repeating unit containing glucose, galactose and rhamnose. The biosynthesis of this polymer depends on both the specific *eps* genes organized in a plasmid-located gene cluster and on several household genes that are involved in biosynthesis of the primary EPS precursors, the nucleotide-sugars. This work focused on the household genes to induce overproduction and/or structural modification of EPS. Therefore, we cloned, characterized, and controlled expression of the genes that encode enzymes involved (i) in primary sugar metabolism (*glk*, *pfk*, *fbp*, *pgm*), (ii) the biosynthetic pathway from glucose-1P to the EPS precursors UDP-glucose (*galU*), UDP-galactose (*galU* and *galE*) and dTDP-rhamnose (*rfbACBD)*, and (iii) in the specific pathway (*epsA-K*) for the assembly of the repeating unit, export and polymerization of the NIZO B40 EPS. We provide evidence for metabolic control of the *gal* and *rfb* genes in EPS precursor and EPS production. Overexpression of the *galU*, *pgm* or the *rfb* genes resulted in a significant increase of EPS-precursors. Moreover, overexpression of the *eps* genes led to four-fold increased NIZO B40 EPS production. In addition, reduction of the UDP-galactose level by *galE* disruption abolished EPS production while a *rfb* conditional knock-out yielded an EPS with altered sugar composition and different physical characteristics.

The research described in this thesis contributes to the understanding of exopolysaccharide biosynthesis in lactic acid bacteria and provides a starting point for applications in the dairy industry, especially with respect to the texture and health benefits of fermented products.

Outline of this Thesis

Since exopolysaccharides (EPS) play an important role in the texture of fermented milk products and are suggested to confer health benefits to their consumers, there is considerable interest in lactic acid bacteria producing these polymers. EPS production in *Lactococcus lactis* NIZO B40 depends on specific *eps* genes encoded on the NIZO B40 EPS-plasmid and on several household genes (*galU*, *galE*, *rfbACBD*) encoded on the *L. lactis* chromosome. The aim of the work described in this thesis is to gain insight in EPS biosynthesis by use of metabolic engineering as a tool to increase EPS production and to alter EPS composition.

In **Chapter 1** an introduction on the impact of engineering sugar catabolism on the biosynthesis of EPS in lactic acid bacteria and a review of current knowledge is given.

Chapter 2 describes the regulation of EPS production by the source of sugar by determining the enzyme activities involved in sugar nucleotide biosynthesis. It was demonstrated that the relative efficiencies of primary sugar conversions were responsible for the difference in EPS production in glucose- and fructose-grown cultures.

Chapter 3 is focussed on the glycolytic branching point of sugar breakdown and sugar nucleotide biosynthesis. Increase of phosphoglucomutase, (Pgm), activity resulted in an approximate four-fold increase in both UDP-glucose and UDP-galactose levels. However, increased activity of Pgm or glucokinase (Glk), or decreased activity of phosphofructokinase (Pfk), did not significantly influence NIZO B40 EPS production levels. These results suggest that the activity level of the Gal, the Rfb or the specific Eps enzymes might control the level of NIZO B40 EPS production.

Chapter 4 reports on the impact of the lactococcal *galU* and *galE* genes on sugar nucleotide and EPS biosynthesis. Overproduction GalU led to an approximate eight-fold increase in both UDP-glucose and UDP-galactose levels, but did not significantly influence NIZO B40 EPS production. These results suggest that the activity level of the Rfb or the specific Eps enzymes might control the level of NIZO B40 EPS production. Disruption of *galE* resulted in poor growth, undetectable UDP-sugar levels, and elimination of EPS production when cells were grown in media with glucose as sole carbon source. Addition of galactose restored growth, but the level of EPS production remained approximately two-fold lower than that in the wild-type strain.

In **Chapter 5** the identification and characterization of the lactococcal *rfbACBD* operon, required for dTDP-rhamnose biosynthesis, is described. It is shown that overproduction of the *rfbACBD* genes led to two-fold increases of dTDP-rhamnose levels, but did not significantly influence NIZO B40 EPS production. These results indicate that the activity level of the specific Eps enzymes might control the NIZO B40 EPS production. Moreover, a *rfb* conditional knock-out yielded an EPS with altered sugar composition and different biophysical characteristics.

Chapter 6 focussed on the effects of increased EPS-plasmid copy number on EPS production. It was shown that an approximately nine-fold increase of the EPS-plasmid number resulted in four-fold increased NIZO B40 EPS production level, indicating that the level of NIZO B40 EPS production is controlled at the level of the specific *eps* genes rather than at the level of the household genes.

Finally, the results of Chapter 2 to 6, combined with additional data are discussed in **Chapter 7** focussing on engineering EPS biosynthesis, including the relation to the control of enzyme activities and practical applications and perspectives of this work.

Chapter 1

Chapter

Sugar Catabolism and its Impact on the Biosynthesis and Engineering of Exopolysaccharide Production in Lactic Acid Bacteria

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Abstract

Over the last years, the production of exopolysaccharides (EPS) by lactic acid bacteria (LAB) has been extensively studied. These EPS play an important role in the rheology and texture of fermented food products. Significant progress in the understanding of EPS biosynthetic pathways, genetics, kinetic models, and physics has been made. This knowledge can now be applied to rationally design metabolic engineering studies to modify EPS production and composition. This mini review will discuss the potential engineering strategies of sugar catabolism for the production of EPS by LAB.

Introduction

In many bacterial species sugar catabolism is not only crucial for the generation of energy and biomass but also for the production of polysaccharides. Microbial polysaccharides can be present as constituents of cell walls, as part of lipo-polysaccharides (LPS), often referred to as O-antigens, as capsular polysaccharides (CPS) covalently associated with the cell surface, or secreted as exopolysaccharides (EPS) into the extracellular environment. While the cell-associated polysaccharides like CPS and LPS are of considerable medical importance since they contribute to the virulence of their producers, many of the EPS have application in the food industry. EPS-producing lactic acid bacteria (LAB), are used to generate the rheological characteristics and texture properties of specific fermented dairy and food products. These food-grade bacteria produce a wide variety of structurally different EPS that are of interest for food applications as *in situ* produced natural biothickeners. However, they also have potential to be applied as ingredients, replacing presently applied stabilizers and thickeners that are produced by non-food grade bacteria (Sutherland, 1998; Becker *et al.*, 1998). Moreover, it has been suggested that some EPS from LAB may confer health benefits to the consumer. Mouse model studies have indicated that EPS may have immunostimulatory (Hosono *et al.*, 1997), antitumoral (Kitazawa *et al.*, 1991) or cholesterol-lowering activity (Nakajima *et al.*, 1992a).

Over the past few years, various studies have been initiated to advance our understanding of the genetics and biochemistry of microbial EPS biosynthesis, and explore the possibilities to produce polysaccharides with desired viscosifying properties. Here we review the present examples and discuss the potential of future possibilities of engineering strategies of sugar catabolism for the production of EPS by LAB, with special attention for *Lactococcus lactis*, which is among the best characterized model system.

Biosynthesis of LAB polysaccharides. EPS-producing LAB, including the genera *Streptococcus*, *Lactobacillus, Leuconostoc* and *Lactococcus*, produce a wide variety of structurally different polymers, in a size that range from 4 $X10^4$ to 6 $X\ 10^6$ Da (de Vuyst and Degeest, 1999). Different classes of EPS can be distinguished based on the mechanism of biosynthesis and the precursors required (Sutherland, 1993). The first class comprises the extracellularly produced homopolysaccharides like dextran, levan, and mutan. The polymerization reaction in these cases proceeds via extracellular glycosyltransferases, which transfer a monosaccharide from a disaccharide to a growing polysaccharide chain (Fig. 1.1). Since their production is rather simple, independent of the central carbon metabolism, and the variations in structure are limited, this class of EPS is not further discussed here. The other categories comprise homo- and heteropolysaccharides with (ir)regular repeating units that are synthesized

from intracellular sugar nucleotide precursors (Fig. 1.1). Some of these sugar nucleotides serve as precursors for EPS biosynthesis. However, they are also involved in the biosynthesis of several cell-wall components and can therefore be considered essential for growth.

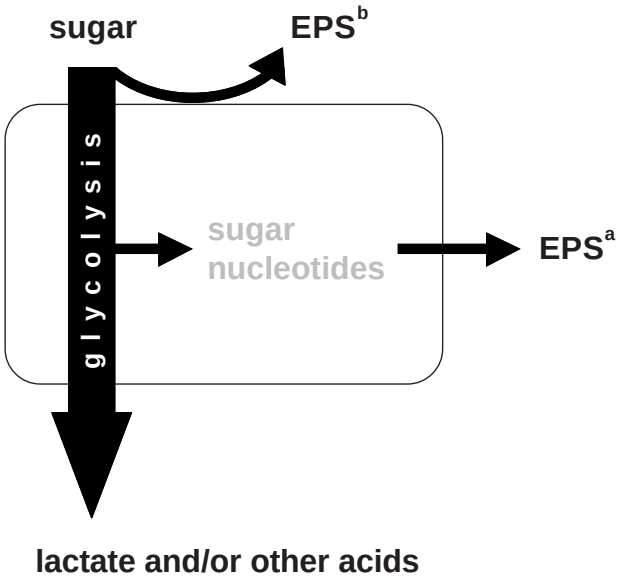


Fig. 1.1. Schematic representation of pathways involved in sugar fermentation via glycolysis to lactate and/or other acids and biosynthesis of exopolysaccharides (EPS) from intracellular sugar nucleotides (EPS[a]) and extracellular EPS biosynthesis (EPS[b]).

The EPS that is produced by LAB from intracellular sugar nucleotides may consist of a variety of sugars including glucose, galactose, rhamnose, glucuronic acid, fucose, *N*-acetylglucosamine (GlcNAc), and *N*-acetyl-galactosamine (GalNAc). These are all formed from intermediates of the central carbon metabolism that starts with the transport of the sugar for which three basically different systems are known within LAB (de Vos, 1996). In primary transport systems, ATP hydrolysis is directly coupled to sugar translocation via a sugar transport ATPase (Fath and Kolter, 1993). In secondary transport systems, the import is coupled to transport of ions and other solutes (Poolman, 1993). Finally, the group translocation system operates in LAB during sugar transport via the phosphoenolpyruvate (PEP) transport system (PTS). In this system a phosphate group, released from the conversion of PEP into pyruvate, is transfered to the incoming sugar involving two general cytoplasmic phosphocarrier proteins, Enzyme I and HPr (Postma *et al.*, 1993). Many sugars are imported in LAB via this latter system which is energetically efficient since the sugar is translocated and phosphorylated in a single step at the expense of one ATP that would otherwise be generated by PEP conversion into pyruvate catalized by pyruvate-kinase. After uptake, sugars are fermented through different pathways (Thompson, 1987). These are integrated in the glycolysis for the subsequent conversion to pyruvate (Fig. 1.2). Pyruvate is then converted to solely lactate or to a mixture of lactate and organic acids. Depending on the mode of transport, the following step in lactose metabolism involves either cleavage of the unmodified sugar by β-galactosidase, generating glucose and galactose, or hydrolysis of lactose-6P by phospho-β-galactosidase, yielding galactose-6P and glucose. Glucose is fermented via the glycolytic pathway or the phosphoketolase pathway (Kandler, 1983). While galactose-6P is fermented by the tagatose-6P pathway, galactose is either metabolized

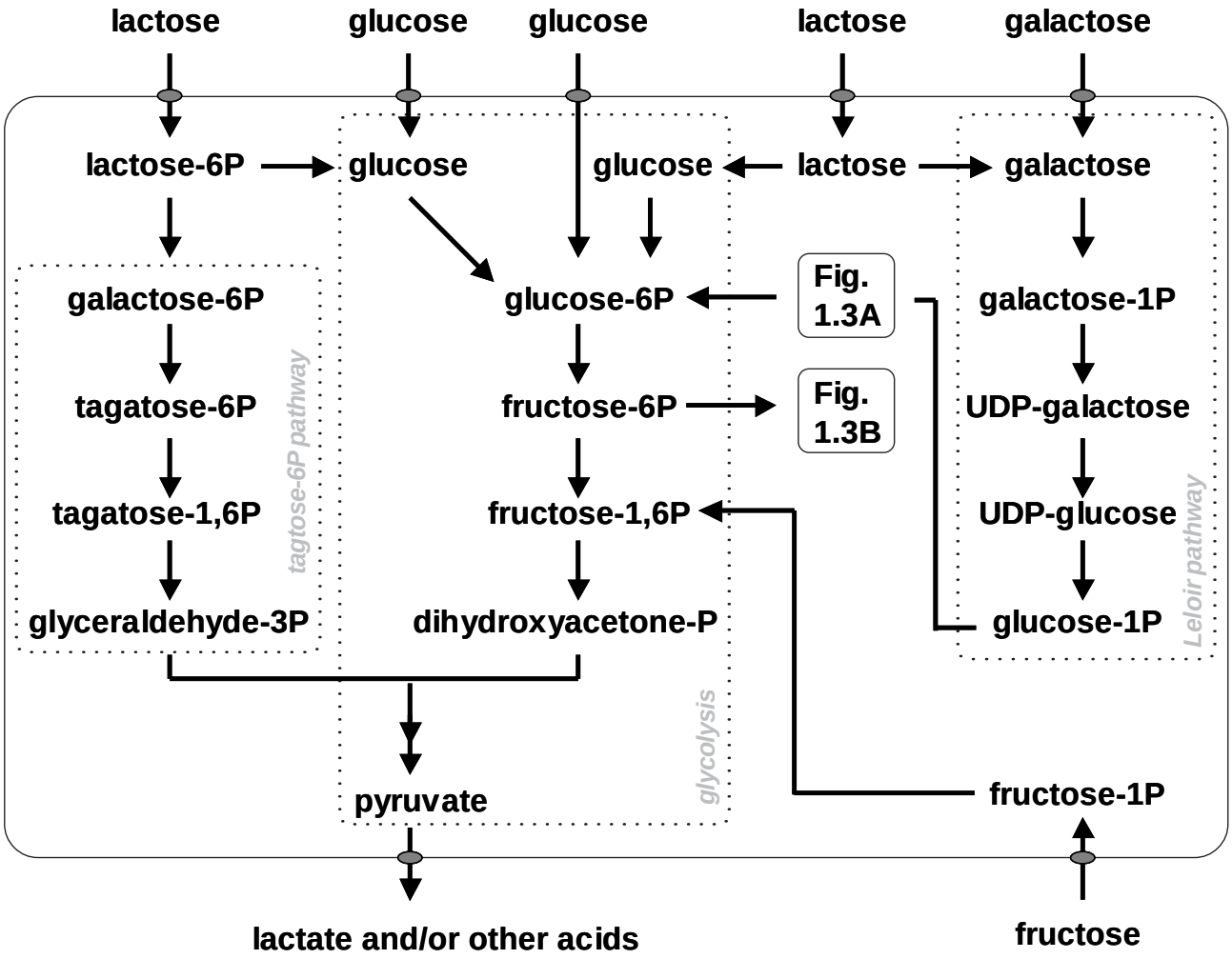
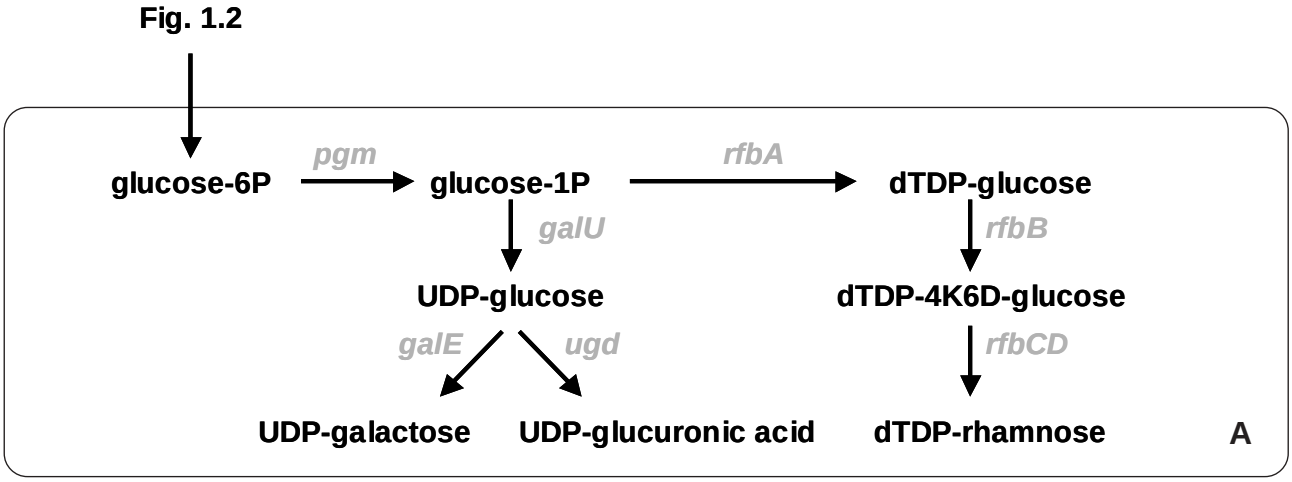


Fig. 1.2. Schematic representation of pathways involved in central sugar metabolism: glycolysis, Leloir pathway, and tagatose-6P pathway are indicated with dotted lines.

via the Leloir pathway by enzymes encoded within one or more *gal* gene clusters (Grossiord *et al.*, 1998), or in some species, like *Streptococcus thermophilus* or *Lactobacillus delbrueckii* subsp. *bulgaricus*, secreted into the medium (Hutkins and Morris, 1987). Similarly, other carbohydrates may enter the cell either in a phosphorylated state or as a free sugar depending on their mode of transport. In the latter case the sugar has to be phosphorylated prior to further degradation. In many of the metabolic pathways leading to the formation of the required sugar nucleotides, glucose-1P is a central metabolite that is formed from the glycolysis intermediate glucose-6P by phosphoglucomutase (Pgm) activity (Ramos *et al.*, 2001) (Fig. 1.3A). Glucose-1P is converted into dTDP-rhamnose by the sequential activities of glucose-1P thymidylyltransferase (RfbA), dTDP-glucose-4,6 dehydratase (RfbB), dTDP-4-keto-6-deoxy-*D*-glucose-3,5 epimerase (RfbC,), and dTDP-4-keto-*L*-rhamnose reductase (RfbD). Alternatively, UDP-glucose pyrophosphorylase (GalU) activity catalyzes the conversion of glucose-1P into UDP-glucose, which subsequently is converted into UDP-galactose, by UDP-galactose epimerase (GalE) activity, or to UDP-glucuronic acid by UDP-glucose dehydrogenase activity (Ugd). A second central intermediate in sugar nucleotides biosynthesis is the glycolysis

intermediate fructose-6P from which UDP-GlcNAc and UDP-GalNAc are formed via the amino-sugars metabolism (Fig. 1.3B). Furthermore, GDP-fucose is formed from fructose-6P via the fructose-mannose metabolism (Fig. 1.3B). Finally, these sugar nucleotides can be assembled into a growing repeating unit by glycosyltransferase activities, exported to the outside of the cell and polymerized, to complete EPS biosynthesis.



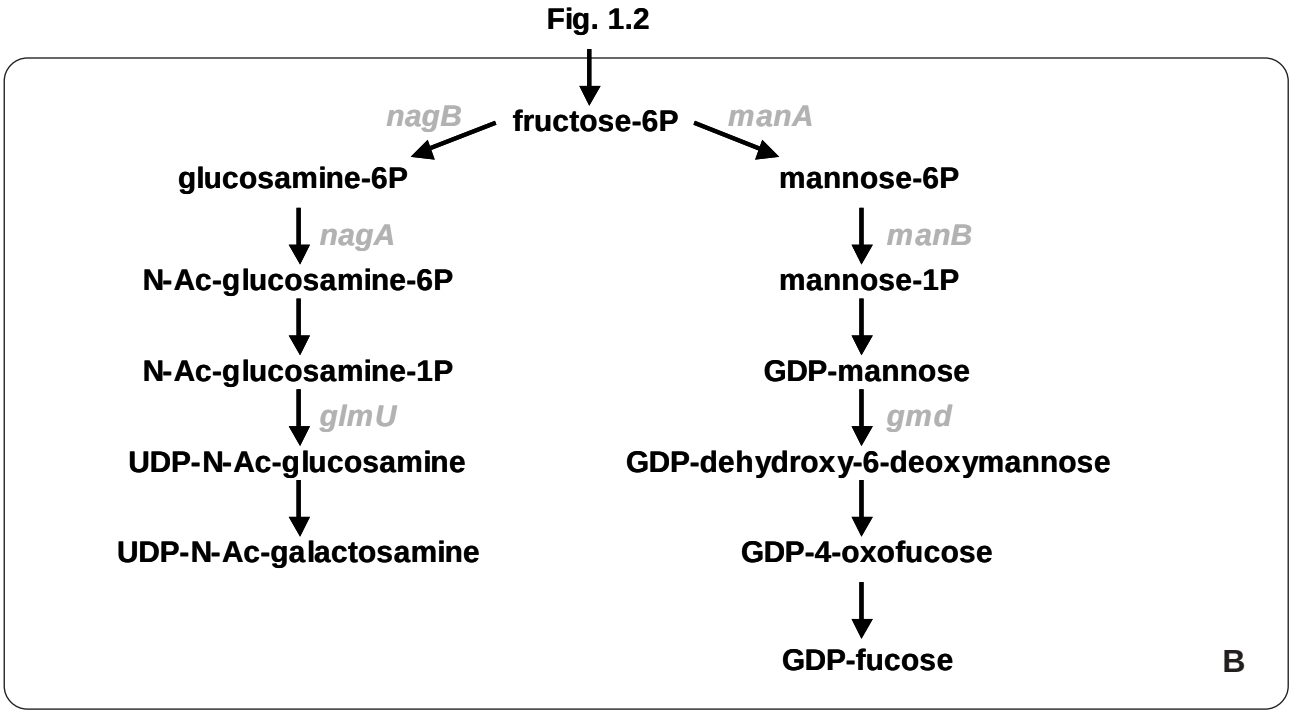


Fig. 1.3. Schematic representation of pathways involved in sugar nucleotide metabolism from glucose-6P (A) and fructose-6P (B). Enzymes involved (encoded genes are indicated): Pgm, phosphoglucomutase; GalU, UDP-glucose pyrophosphorylase; GalE, UDP-galactose epimerase; Ugd, UDP-glucose dehydrogenase; RfbA, glucose-1P thymidylyltransferase; RfbB, dTDP-glucose-4,6 dehydratase; RfbC, dTDP-4-keto-6-deoxy-*D*-glucose-3,5 epimerase; RfbD, dTDP-4-keto-*L*-rhamnose reductase. NagB, glucosamine-6P isomerase; NagA, N-acetylglucosamine-6P deacetylase; GlmU, UDP-N- acetylglucosamine pyrophosphorylase; ManA, mannose-6P isomerase; ManB, phosphomannomutase; Gmd, GDP-mannose-4,6 dehydratase (http://www.kegg.genome.ad.jp/).

Genetics of EPS biosynthesis by LAB. The EPS-producing phenotype was likely to be plasmid-associated in *Lactobacillus casei* and *L. lactis* (Vescovo *et al.*, 1989; von Wright and Tynkkynen, 1987; Vedamuthu and Neville, 1986). This was corroborated by the observation that transfer of several of the lactococcal EPS-plasmids resulted in an EPS-producing phenotype of the recipient strain (van Kranenburg *et al.*, 1997; von Wright and Tynkkynen, 1987; Vedamuthu and Neville, 1986). However, the discovery of the structural genes involved in EPS production provided the first direct evidence for the plasmid location of EPS production (van Kranenburg *et al.*, 1997, 1999a). In contrast, for thermophilic yoghurt bacteria, EPS production was found to be encoded by the chromosome (Lamothe, 2000; Almiron-Roig *et al.*, 2000; Stingele *et al.*,1996). In all cases, genes involved in EPS biosynthesis are organized in gene clusters that show significant conservation in organization and sequence. The organization of these gene clusters directing EPS biosynthesis of *L. lactis* NIZO B35, B40, B891 (van Kranenburg *et al.*, 1997, 1999b), *S. thermophilus* Sfi6 (Stingele *et al.*, 1996; Stingele *et al.*, 1999a), *S. thermophilus* NCFB 2393 (Almiron-Roig *et al.*, 2000), and *Lactobacillus delbrueckii* subsp. *bulgaricus* Lfi5 (Lamothe, 2000) are highly comparable with *cps* gene clusters in *Streptococcus pneumoniae* (Kolkman *et al.*, 1997; Morona *et al.*, 1997) and *Streptococcus agalactiae* (Yamamoto *et al.*, 1999), which are involved in CPS biosynthesis of these organisms. These gene clusters encode enzymes that are involved in the formation of repeating unit oligosaccharides by the sequential addition of sugars to a membrane-anchored lipid carrier as well as enzymes that are putatively involved in export and polymerization of these lipid-linked oligosaccharides. One of the best characterized *eps* gene clusters of LAB strains is that of *L. lactis* NIZO B40 (for a recent review see Kleerebezem *et al.*, 1999). This strain produces a polymer with a regular repeating unit, →4)[α-*L*-Rha*p*-(1→2)][α-*D*-Gal*p*-1-PO_4-3]-β-*D*-Gal*p*-(1→4)-β-*D*-Glc*p*-(1→4)-β-*D*-Glc*p*-(1→, which is structurally identical to that produced by *L. lactis* SBT 0495 (van Kranenburg *et al.*, 1997; Nakajima *et al.*, 1992b). *L. lactis* NIZO B40 strain harbors a 42,180 bp EPS-plasmid pNZ4000 (van Kranenburg *et al.*, 2000), containing the 12 kb *epsRXABCDEFGHIJKL* operon (Fig. 1.4). Based on sequence comparisons most *eps* genes involved in polysaccharide biosynthesis could be annotated (Fig. 1.5.). The putative product of the first gene *(epsR)* shows significant homology with regulator proteins. Interestingly, similar possible regulator genes were found in *S. thermophilus* Sfi6 (Stingele *et al.*, 1996), *S. thermophilus* NCFB 2393 (Almiron-Roig *et al.*, 2000) and *Lb. delbrueckii* subsp. *bulgaricus* Lfi5 (Lamothe, 2000). Although it has been suggested that these *epsR* gene products could be involved in regulation of the expression

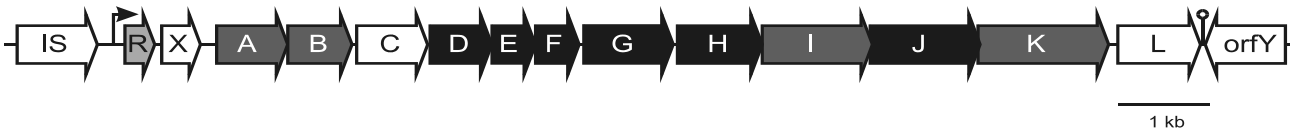


Fig. 1.4. Physical map and genetic organization of the NIZO B40 *eps* gene cluster. Gene products are characterized based on homology studies with gene products involved in polysaccharide biosynthesis (see text). EpsDEFG have been functionally shown to be involved in the assembling of the repeating backbone.

of the corresponding *eps* gene cluster, no experimental evidence supporting this hypothesis has been reported to date. The gene products of *epsDEFGH* and *epsJ* are predicted to act as glycosyltransferases that link sugar nucleotides to a growing repeating unit. EpsI and EpsK are homologous to polymerase and export proteins, respectively, involved in O-antigen production in Gram-negative bacteria (Liu *et al.*, 1996; Macpherson *et al.*, 1995; Morona *et al.*, 1994; Brown *et al.*, 1992). EpsA and EpsB share similarity to an ExoP-like family of proteins that is supposed to be involved in chain length determination (Becker *et al.*, 1995).

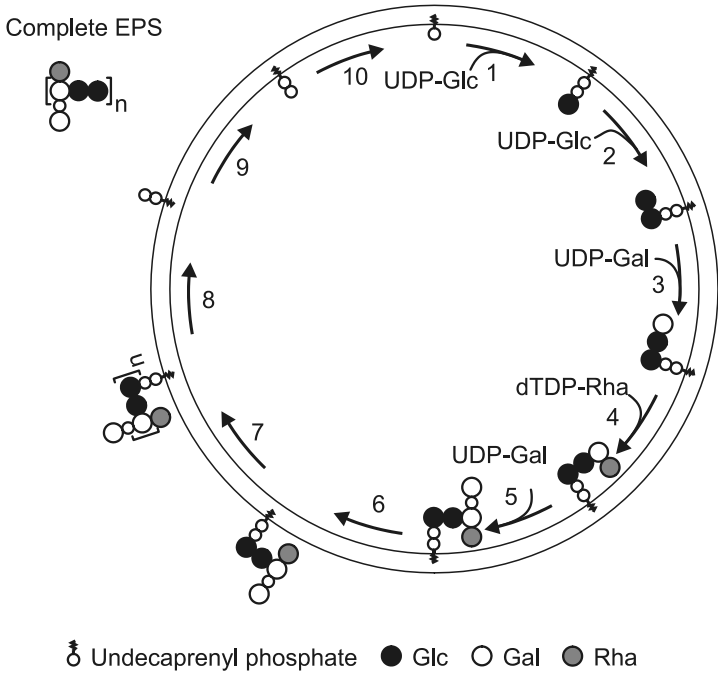


Fig. 1.5. Working model for NIZO B40 EPS biosynthesis at the lactococcal membrane. (1) EpsD links glucose-phosphate from UDP-glucose (UDP-Glc) to the lipid carrier. (2) EpsE and EpsF add the second glucose moiety. (3) EpsG adds galactose from UDP-galactose (UDP-Gal). The repeating unit is completed by the addition of rhamnose (Rha) from dTDP-rhamnose (dTDP-Rha) and galactose-phosphate from UDP-Gal, and EpsH (4) and EpsJ (5) are expected to be involved in these steps. Repeating units are predicted to be translocated accross the membrane by activity of EpsK (6) and subsequently polymerized by EpsI, with EpsA and EpsB determining the chain-length (7, 8). The lipid carrier is retranslocated (9) and dephosphorylated (10).

Functional analysis by homologous and heterologous expression of the glycosyltransferase genes confirmed this and showed that biosynthesis of the *L. lactis* NIZO B40 polysaccharide backbone is initiated by the linkage of a glucose from UDP-glucose to a lipid-carrier by the priming glucosyltransferase EpsD (van Kranenburg *et al.*, 1997). Subsequently, the combined activity of EpsE and EpsF link glucose to the lipid-linked glucose generating a lipid-linked cellobiose. Finally, EpsG links galactose to the lipid-linked cellobiose, completing the backbone of the B40-EPS repeating unit. It is likely that subsequent steps of the repeating unit synthesis include the coupling of the side-chain sugars rhamnose and galactose-phosphate to the galactose moiety in the backbone. These are suggested to be catalyzed by EpsH and EpsJ, respectively (van Kranenburg *et al.*, 1997, 1999a, 1999b). Remarkably, Oba *et al.* (1999) reported a different repeating unit assembly of the related *L. lactis* SBT 0495 EPS based on the identification of several lipid-linked sugar moieties obtained from mild hydrolysis of lipid extracts. However, that there are different biosynthetic pathways for *L. lactis* NIZO B40 and SBT 0495 EPS is not very likely as the *eps* gene-clusters are similar and the EPS structures are identical for the two strains (van Kranenburg *et al.*, 1997, unpublished results). Since the intermediates were identified from

a pool of EPS and cell wall sugar intermediates, it remains to be established whether the identified sugar moieties are true EPS biosynthesis intermediates.

The genes involved in sugar-nucleotide precursor biosynthesis are encoded on the *L. lactis* chromosome. Despite the obvious importance of sugar-nucleotide biosynthesis for the optimal production of EPS by LAB, its genetics has only recently received attention in case of *L. lactis*. This resulted in the identification of the *L. lactis* MG1363 *galU* gene, the gene product of which is involved in the conversion of glucose-1P into UDP-glucose (Boels *et al.*, 2001). In the same strain, the Leloir enzyme GalE involved in the interconversion of UDP-glucose into UDP-galactose was found to be encoded by the *galE* gene, which is located in the *galAMKTE* operon (Grossiord, 1998), whereas the genes for Rfb enzymes involved in the biosynthesis of dTDP-rhamnose from glucose-1P are located within the *rfbACBD* operon (Boels *et al.*, 2002a).

Structural engineering of exopolysaccharides. Detailed analysis of the structure-function relation of polysaccharides related to their physical properties might allow the prediction of the thickening efficiency of a specific (novel) EPS. The intrinsic viscosity is the key-parameter that describes the general concentration dependence of any flexible polysaccharide. This parameter depends on the combination of the polymer size, usually expressed as the radius of gyration, and the molar mass. The relation between the radius of gyration and the molar mass is determined by the chain stiffness which depends on sugar composition, type of linkages, charged groups, and degree of branching (Tuinier *et al.*, 1999, 2001). Structure engineering aims at altering the physical properties of the polymers by changing their primary structure. To gain insight in these structure-function relationships, closely related structures need to be studied. These can be generated following polymer synthesis by various treatments of the native structure, here designated “post-synthetic engineering”. Alternatively, engineering of EPS is possible at the level of biosynthesis and allows for genetic approaches to influence the structure composition. A simple post-synthetic engineering method to obtain EPS with an altered structure is its modification by means of enzymatic treatments, an approach that has mainly been used to elucidate chemical structures (Rudd *et al.*, 1997). The most common sources of EPS-degrading enzymes are micro-organisms or their bacteriophages. The best described enzymes are the polysaccharide hydrolases and lyases which can either act endogenously or exogenously. Endoglycosidases hydrolyze the polymer backbone or extended side chains resulting in a fast decrease of the polymerization degree, while exoglycosidases hydrolyze the terminal ends of the backbone or side chains resulting in a slower decrease of the polymerization degree (Sutherland, 1999). Recently, a few LAB polymer structures were subjected to enzymatic treatment resulting in altered structures with modified physical properties (Tuinier *et al.*, 2001). From *L. lactis* B39 and B891 EPS the galactosyl terminal end could be removed by degalactosylation. Subsequent evaluation of the biophysical properties suggested that the thickening efficiency of these modified polymers was negatively affected by this modification (Tuinier *et al.*, 2001). Enzymatic treatment alone might not always be an effective method to alter polymer structures.

For example, the presence of acyl-group substituents strongly inhibits some of the polysaccharide lyases including alginate and gellan lyases (Sutherland, 1999). Moreover, some EPS molecules display an extremely high resistance to enzymatic degradation. A clear example is *L. lactis* B40 EPS that appeared resistant to degradation by a large variety of enzyme preparations (Ruijssenaars *et al.*, 2000).

Post-synthetic engineering approaches by using mild chemical degradation procedures such as *O*-deacetylation by ammonium hydroxide (Robijn *et al.*, 1995) or dephosphorylation using hydrofluoric acid (Nakajima *et al.*, 1992b) can be applied to all EPS and have been used to obtain modified EPS structures. It has been shown that in some cases the presence or absence of functional groups such as acyl- or pyruvyl- groups can have a great impact on physical properties of the polymers (for review see Sutherland, 1997). For example, removal of *O*-acetyl moieties and absence of pyruvate moieties, situated in the side chains of xanthan polymers, positively influences the strength of the gel network formed with the modified polymers (Sutherland, 1997). In contrast, removal of *O*-acetyl groups of *L. lactis* NIZO B891 EPS did not significantly influence the thickening efficiency (Tuinier *et al.*, 2001). Although chemical treatment is mostly applied to elucidate polysaccharide structures, it could be a suitable method to alter side chain structures of the polymer, aiming at improving its application potential. This could be a particularly interesting approach when polysaccharide-modifying enzymes are not available. The use of a combined chemical and enzymatic approach is illustrated by degradation of *L. lactis* NIZO B40 EPS which was insensitive to enzymatic degradation (Ruijssenaars *et al.*, 2000). When the galactosyl side chain of this EPS was removed through chemical treatment, some enzymes were able to degrade the degalactosylated EPS. Furthermore, upon additional partial removal of the rhamnosyl side chain through chemical treatment, the enzymatic degradation could proceed even further (van Casteren *et al.*, 1998). Moreover, also enzymatic degalactosylation of *L. lactis* B891 EPS was more effective when the EPS was first chemically *O*-deacetylated (van Casteren *et al.*, 2000). These results imply that a combined approach of chemical and enzymatic treatment may enhance the ability to obtain desired structures.

There is a growing interest in genetic approaches that affect EPS biosynthesis, as an alternative to the post-synthetic engineering strategies as described above. Genetic engineering of specific genes that are involved in side chain synthesis of xanthan gum has resulted in variants with differences in acetylation and/or pyruvylation or with truncated side chains that had changed viscosifying properties compared to wild-type xanthan (Hassler and Doherty, 1990). Furthermore, random mutagenesis of *Lactobacillus sakei* 0 -1 has yielded a strain that produces an EPS with different monosaccharide composition (Breedveld *et al.*, 1998). However, no genetic defect could be identified in these mutants, and therefore the explanation of the observed effect remains unclear. Encouraging results have been obtained by heterologous expression of polysaccharide gene clusters (Stingele *et al.*, 1999b; Pollock et al, 1997). Stingele *et al.* (1999b) reported that expression of the *S. thermophilus* Sfi6 *eps* gene cluster in *L. lactis* MG1363 resulted in EPS production. The resulting strain produced a new EPS with molecular mass similar to that of the

Sfi6 EPS (≥ 2 X10^6 Da), but with an altered repeating unit. This novel EPS contained a galactose moiety instead of a GalNAc moiety in the repeating unit backbone and lacked the galactose side chain moiety. This is probably caused by the inability of *L. lactis* to produce UDP-GalNAc resulting in incorporation of galactose instead of GalNAc into the backbone. It is possible that the lack of the side chain moiety then results from the specificity of the *S. thermophilus* side chain galactosyltransferase that may rely on the presence of the *N*-acetylgroup of the neighbouring GalNAc moiety in the backbone in order to be functional. If so, the glucosyl-transferase, required for the incorporation of glucose moiety into the backbone does not have such specificity for the acetyl-group, since glucose is linked to the galactose moiety in the backbone of the new EPS. These results suggest that the glycosyltransferases have no exclusive selectivity for donor and acceptor sugar moieties while the polymerization and export machinery may not exhibit exclusive specificity for the native repeating unit (Stingele *et al.*, 1999b). The increasing numbers of glycosyltransferases characterized (Jolly and Stingele, 2001) provide tools for new poly- or oligo-saccharide engineering approaches by introducing new or existing glycosyltransferases in EPS-producing LAB and other bacteria. A first example of such an approach is the possibility of functionally exchanging glycosyltransferases with identical substrate specificity originating from different genera in Gram-negative (Pollock *et al.*,1997) and Gram-positive bacteria (van Kranenburg *et al.*, 1999a). These findings, in combination with the possibilities raised by the heterologous expression of Sfi6 *eps* gene cluster (see above), suggest that a combined approach may allow the construction of engineered *eps* gene clusters that direct the biosynthesis of oligomers and even polymers of a chosen structure in a host of interest.

Engineering of EPS production. Microbial food additives should be preferentially produced by food-grade bacteria. Microbial EPS like xanthan that is produced by the non-food grade *Xanthomonas campestris*, is widely applied as thickening, gelling and stabilizing ingredients in the food industry (Sutherland, 1998). LAB, have a long history of safe use in foods are excellent sources of EPS. Although the EPS production levels in LAB are relatively low (40-800 mg l^{-1}) compared to xanthan production level in *X. campestris* (10-25 g l^{-1}) (Becker *et al.*, 1998), the EPS from LAB are effective thickeners (Hess *et al.*, 1997). Studies aimed at engineering EPS structure (see above) hold a promise for the directed engineering of rheological properties of EPS. Unfortunately, the modified LAB polymers described to date are only formed at a fraction of that produced by the wild-type strains (Stingele *et al.*, 1999b). Therefore, bottlenecks in the physiology and genetics of LAB in relation to EPS production levels have to be identified before native and modified EPS could be of economical interest for the use as food ingredients.

Insight in the biological role of EPS could provide a rationale for the design of strategies to create physiological conditions leading to improved EPS production. Alternatively, this knowledge might allow the design of conditions to select for mutants with increased EPS production levels. However, the physiological function of EPS has not been established clearly. It is not likely that EPS serve as a reserve sources of energy and carbon since EPS-producing

bacteria are mostly not capable of degrading their own EPS, although unrelated microbes sometimes catabolize polysaccharides of other bacteria (Dudman, 1977). Remarkably, the EPS-producing ability does not seem to be essential since the capacity to produce EPS is not very stable and physical or enzymatic removal of EPS does not negatively affect cell growth *in vitro* (Schellhaass, 1983). Several other biological roles of EPS have been suggested. These include a selective advantage in the natural habitat of these microbes, such as protection against dehydratation, phagocytosis, predation by protozoa, bacteriophage attack, antibiotics, or toxic compounds (Roberts, 1996; Weiner *et al.*, 1995; Cerning, 1990). It has also been suggested that EPS play a role in the sequestering of essential cations (Looijesteijn *et al.*, 2001; Weiner *et al.*, 1995), in adhesion and in biofilm formation (Roberts, 1996). For *L. lactis* it was reported that cell-associated EPS resulted in protection of the cell against bacteriophage attack (Looijesteijn *et al.*, 2001; Forde and Fitzgerald, 1999). However, no measurable increase in EPS production could be realized in *L. lactis* by using bacteriophages as inducing factors (Looijesteijn *et al.*, 2001).

The amount of EPS that is produced by LAB depends on the physiological conditions used. Under non-optimal laboratory conditions, EPS production by LAB appeared to be an unstable property, which is manifested by genetic instability. This instability may be the result of genomic instability caused by deletions or rearrangements within the *eps* gene cluster or by loss of an entire EPS-plasmid. Numerous studies on the influence of environmental conditions or medium composition were performed to optimise EPS production in LAB (for a recent review see de Vuyst and Degeest, 1999). Unfortunately, from the variable and sometimes contradictory results reported for different strains it can be concluded that there is no single set of culture conditions that results in optimal EPS yields for all LAB. However, some indications of controlling steps for EPS production by LAB were obtained from studying the physiological conditions that influence the production level. Promissing results have been obtained with *L. lactis* NIZO B40 where growth on fructose produced much less EPS compared to cells grown on glucose. The production of EPS on fructose could be elevated by the functional overproduction of fructose-1,6 diphosphatase (Looijesteijn *et al.*, 1999). This enzyme is required for the conversion of fructose-1,6P into fructose-6P, which in fructose metabolism is an essential step for the generation of glucose-1P, which is the central sugar nucleotide precursor for B40 EPS biosynthesis (Fig. 1.2, 1.3A).

It is relevant for metabolic engineering strategies that aim at increased fluxes to include physiological and genetic control factors in sugar degradation and EPS formation pathways (de Vos, 1996). A possible control factor in EPS biosynthesis is the availability of the required sugar nucleotides. A possible key step in the control of sugar nucleotide biosynthesis is the interconversion of the glycolysis intermediate glucose-6P into the sugar nucleotide precursor glucose-1P, catalyzed by Pgm (Fig. 1.3A). To demonstrate this concept, the *E. coli* α-*pgm* gene was overexpressed in *L. lactis* resulting in highly increased levels of the UDP-glucose and UDP-galactose sugar nucleotides, although the *L. lactis* NIZO B40 EPS production was not significantly

affected (Boels *et al.*, 2002b). An alternative approach was recently described and involves the overexpression of *cps3D* (Ugd analogue, Fig. 1.3A) and *cps3S* (type 3 CPS synthase) genes from *S. pneumoniae* type 3 in *L. lactis* (Gilbert *et al.*, 2000). This resulted in a low level production of type 3 CPS by the *L. lactis* strain that could be strongly increased by co-expression of the *cps3U* gene that encodes a GalU analogue (Gilbert *et al.*, 2000). This indicates that the level of UDP-glucose in lactococcal cells is apparently limited by the endogenous GalU enzyme level. Functional homologous overexpression of the *galU* gene in *L. lactis* resulted in increased UDP-glucose and UDP-galactose levels, supporting the notion that the GalU enzyme activity indeed controls the production of these sugar nucleotides in wild-type cells (Boels *et al.*, 2001). However, this increased precursor availability did not lead to a higher EPS production in *L. lactis* NIZO B40. Another essential enzyme in sugar nucleotide and EPS biosynthesis is the Leloir enzyme GalE. Evaluation of EPS production in a *L. lactis galE* mutant strain revealed undetectable levels of UDP-galactose and abolished EPS biosynthesis when cultured on glucose, while EPS production could be recovered by the addition of galactose to the medium. Moreover, this mutant was also affected in cell division when grown on glucose alone, which indicates a critical role for UDP-galactose as a precursor in cell wall biosynthesis (Boels *et al.*, 2001; Grossiord, 1998). Finally, the availability of dTDP-rhamnose, which can be incorporated on the side chain, could limit *L. lactis* NIZO B40 EPS production (Boels *et al.*, 2002a). Data supporting that dTDP-rhamnose biosynthesis is important in biosynthesis of rhamnose-containing polymers were reported for *rfb* mutants of *S. pneumoniae* (Morona *et al.*, 1997) and *Streptococcus mutans* (Tsukioka *et al.*, 1997), which showed a loss of polymer production.

It is feasible that the production level of EPS in some cases may be determined by the activity of the specific EPS biosynthesis machinery rather than by the level of sugar nucleotides (Boels *et al.*, 2002c). This possibility is supported by the overexpression of the priming glycosyltransferase *epsD* in *L. lactis* which resulted in slightly increased EPS production (van Kranenburg *et al.*, 1999a).

To design metabolic engineering approaches, modelling of the carbohydrate metabolism, including EPS production might be helpful. Metabolic control analysis (MCA) can disclose control points that have a major effect on a particular carbon flux (Holms, 1996; Galazzo and Bailey, 1990). Subsequently, MCA in combination with kinetic modelling studies might facilitate the choice of the most efficient metabolic engineering strategy to increase EPS production. The modelling of carbon-metabolism and experimental studies of the regulation of the glycolytic flux in *L. lactis* are currently in progress (Hoefnagel *et al.*, 2000).

Concluding remarks

The recent advances in analysis of physiology and genetics of LAB producing EPS allow control of their metabolic activity not only by changing the fermentation conditions but also by applying genetic techniques that result in the overexpression of existing or novel genes. The first results of the engineering of EPS structures has shown that expression of *eps* genes in heterologous hosts may result in the production of polymers with modified repeating units. In addition, initial steps to address the metabolic cause of the low production levels of EPS suggest that increased EPS precursor availability, in combination with elevated enzyme activity levels involved in the specific EPS biosynthesis pathway, might enhance EPS production.

At present, the rapid developments in the generation and analysis of whole and partial genome sequences is expected to produce valuable information that should be followed up by further genetic, biochemical and physiological analysis. Combined with MCA this information can be exploited for developing metabolic engineering strategies to produce tailor-made oligo- and polysaccharides for specific applications.

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Chapter 2

Chapter

Regulation of Exopolysaccharide Production by *Lactococcus lactis* subsp. *cremoris* by the Sugar Source

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Abstract

Lactococcus lactis produced more exopolysaccharide (EPS) on glucose than on fructose as sugar substrate, although the transcription level of the *eps* gene cluster was independent of the sugar source. A major difference between cells grown on the two substrates was the capacity to produce sugar nucleotides, the EPS precursors. However, the activities of the enzymes required for the synthesis of nucleotide sugars were not changed upon growth on different sugars. The activity of fructosebisphosphatase (FBPase) was by far the lowest of the enzymes involved in precursor formation under all conditions. FBPase catalyses the conversion of fructose-1,6-diphosphate into fructose-6-phosphate, which is an essential step in the biosynthesis of sugar nucleotides from fructose but not from glucose. By overexpression of the *fbp* gene, which resulted in increased EPS synthesis on fructose, it was proven that the low activity of FBPase is indeed limiting not only for EPS production but also for growth on fructose as a sugar source.

Introduction

Lactic acid bacteria are widely used in the food industry, mainly for lactic acid formation but also for the production of minor food components important for structure, flavour or preservation. Several lactic acid bacteria are able to produce exopolysaccharides (EPS). These EPS- forming bacteria play a considerable role in the rheology and texture of fermented milks. EPS-producing starter cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, for example, are used for yoghurt manufacture in order to improve the viscosity and to prevent syneresis and gel fracture. Furthermore, the presence of mucoid *Lactococcus lactis* subsp. *cremoris* strains in starter cultures for the production of the Scandinavian ropy sour milks Viili and Långfil is essential for the desired textures of these products (Cerning, 1990). Polysaccharides produced by lactic acid bacteria also provide a source of stabilising, viscosifying, emulsifying, gelling, or water binding agents for use as natural additives in various food products, which may be an alternative to texturising agents of plant or animal origin (van den Berg *et al*., 1995).

The strain, the culture conditions, and the medium composition influence the amount of microbial EPS that is produced by a certain species. The type of carbon source has a huge influence on EPS productivity and may also affect the composition of EPS. *Lb delbrueckii* subsp. *bulgaricus* NCFB 2772 produces three times more EPS with glucose than with fructose as a sugar source, and the type of EPS produced by this organism is influenced by the sugar source as well (Grobben *et al*., 1997). The yields of EPS produced by *Lactobacillus casei* CG11, *Lactobacillus rhamnosus* C83, and *S. salivarius* subsp. *thermophilus* are also significantly influenced by the carbon source (Cerning *et al*., 1994; Gancel and Novel, 1994; Gamar *et al*., 1997). In an earlier report we described that *L. lactis* subsp. *cremoris* NIZO B40 produces about nine times more EPS with glucose than with fructose as a sugar source under acidifying conditions (Looijesteijn and Hugenholtz, 1999).

Biosynthesis of polysaccharides that are produced by lactococci starts with the intracellular formation of EPS precursors, the sugar nucleotides, followed by the formation of a repeating unit on a lipid carrier, which is located in the cytoplasmic membrane. The repeating unit of EPS produced by *L. lactis* NIZO B40 is composed of glucose, galactose, rhamnose, and phosphate in a ratio of 2 : 2 : 1 : 1 (van Kranenburg *et al*., 1997; van Casteren *et al*., 1998). The sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose are the donors of monomers for the biosynthesis of this pentasaccharide unit. The last steps of EPS formation most likely involve transport of the repeating units across the membrane to the outer layer and polymerisation of several hundred to several thousand repeating units to form the final EPS (Cerning, 1990; Sikkema and Oba, 1998).

The formation of sugar nucleotides and the use of a lipid carrier are not unique to EPS biosynthesis; both are also involved in the formation of cell-wall sugars (Sutherland, 1982). Enzymes necessary for the other reactions involved in the biosynthesis of EPS by lactococci

are specific and their genes are encoded on an EPS plasmid. EPS production by strain NIZO B40 is encoded by a 12 kb region containing 14 genes with the order *epsRXABCDEFGHIJKL* from the 40-kb EPS plasmid called pNZ4000 (van Kranenburg *et al*., 1997). The *eps* gene cluster is transcribed from a single promoter upstream of *epsR* (van Kranenburg *et al*., 1997). The gene products EpsD, -E, -F, and -G are glycosyltransferases required for synthesis of the EPS backbone (van Kranenburg *et al*., 1999).

Regulation of EPS production may be possible at all the different steps involved in its biosynthesis. We determined the steps during which the sugar source influences the final EPS yield of *L. lactis.*

Materials and methods

Bacterial strains, culture conditions and analysis of growth and product formation. Bacterial strains and plasmids used in this study are listed in Table 2.1. Fermentations with *L. lactis* were performed in a chemically defined medium (CDM) at 30°C and pH 5.8 as described before (Looijesteijn and Hugenholtz, 1999). For fermentations without pH control, 1.9 g of β-glycerophosphate per litre was added to the medium and the concentration of the sugar source was reduced to 5 g l^{-1}. For leucine-limited growth in chemostat cultures, the concentration of leucine was reduced to 30 mg l^{-1}. *Escherichia coli* was grown in tryptone yeast extract (TY) broth with aeration at 37°C. If appropriate, the media contained chloramphenicol (10 mg l^{-1}) and erythromycin (5 mg l^{-1}). Cell growth was determined by measuring the optical density of the culture fluid at 600 nm (OD_{600}). The amount of residual sugars was quantified by high-performance liquid chromatography (HPLC) (van Riel and Olieman, 1991). Organic acids were analysed by HPLC with a Rezex Organic Acid column (Phenomenex Inc., Torrance, Calif.) at 60°C with 0.6 ml min^{-1} of 5 mM H_2SO_4 as the eluent and detection based on a refractive index. The amount of EPS was measured in duplicate by gel permeation chromatography with dextran as the standard as described previously (Looijesteijn and Hugenholtz, 1999). The standard deviation of this method was 2%.

Preparation of cell (free) extracts. Bacteria were harvested by centrifugation (16,000 x *g*, 30 min, 4°C) at an OD_{600} of 1 to1.5, washed twice with 0.85% NaCl, and suspended in 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (Petit *et al*., 1991). The bacteria were disrupted ultrasonically (20 kHz) at 0°C for 36 cycles of 5 s (XL2020 sonicator; Heat Systems, New York, N.Y.). Cell debris was removed by centrifugation (13,000 x *g*, 10 min, 4°C). The protein content of the cell-free extract (CFE) was determined by the method of Bradford (Bradford, 1976). For the assays of the phosphoelnolpyruvate (PEP)-glucose-phosphotransferase system (PTS), the PEP-fructose-PTS, UDP-glucose pyro-

phosphorylase, and dTDP-glucose pyrophosphorylase cell debris was not removed because these enzymes are probably linked to the cell membranes.

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics[a]	Reference
Strains		
L. lactis		
NIZO B40	Lac^+ Eps^+	(van Kranenburg *et al*., 1997)
MG1614	Rf^r,Sm^r, plasmid free	(Gasson, 1983)
NZ4010	MG1614 derivative containing pNZ4000	(van Kranenburg *et al*., 1997)
NZ9000	MG1363; *pepN::nisRK*	(Kuipers *et al*., 1998)
E. coli MC1061		(Casadaban and Cohen, 1980)
Plasmids		
pNZ4000	42-kb plasmid encoding EPS production	(van Kranenburg *et al*., 1997)
pNZ4030	Ery^r, 27-kb derivative of pNZ4000	(van Kranenburg *et al*., 1997)
pNZ4040	Cm^r, plasmid with the marker gene *gusA* under control of the *eps* promoter	(van Kranenburg *et al*., 1997)
pNZ8048	Cm^r, lacococcal cloning and expression vector with the *nisA* promoter upstream of a multiple cloning site	(Kuipers *et al*., 1998)
pNZ4150	pNZ8048 derivative containing the *E. coli fbp* gene translationally fused to the *nisA* promoter	This work

[a]) Lac^+, lactose-fermenting phenotype; Eps^+, exopolysaccharide-producing phenotype; Rf^r, rifampicin resistant; Sm^R, streptomycin resistant; Ery^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

Enzyme assays. Enzyme assays were performed at 30°C in a total volume of 1 ml with freshly prepared cell (free) extracts. The formation or consumption of NAD(P)H was determined by measuring the change in the absorbance at 340 nm. Values are the means of results from at least two independent duplicate measurements. The blank contained the reaction buffer, the cofactors, and the substrate but lacked the cell (free) extract.

The PEP-PTS uptake systems for glucose and fructose were assayed with a mixture containing 50 mM KPO_4 buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM PEP, 0.5 mM NADH, 4 U of lactate dehydrogenase, and cell extract. The reaction was started by adding 1 mM glucose or fructose (Grobben *et al*., 1996).

The reaction mixtures for 1- and 6-phosphofructokinase (EC 2.7.1.56 and EC 2.7.1.11) contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 1.25 mM ATP, 0.15 mM NADH, 4.5 U of aldolase, 18 U of triose-phosphate-isomerase, 6.2 of U glycerol-3-phosphate dehydrogenase, and CFE. Addition of 5 mM fructose-1-phosphate or fructose-6-phosphate started the reactions (Grobben *et al*., 1996).

The reaction mixture for the α-phosphoglucomutase (EC 2.7.5.1) assay contained 50 mM triethanolamine buffer (pH 7.2), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 50 μM glucose-1,6-diphosphate,

4 U of glucose 6-phosphate dehydrogenase, and CFE. The reaction was started by the addition of 1.4 mM α-glucose 1-phosphate (Qian *et al*., 1994).

The phosphoglucose isomerase (EC 5.3.1.9) reverse-reaction mixture contained 50 mM potassium phosphate buffer (pH 6.8), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 4 U of glucose-6-phosphate dehydrogenase, and CFE. The reaction was started by adding 5 mM fructose-6-phosphate (Grobben *et al*., 1996).

The UDP-galactose-4-epimerase (EC 5.1.3.2) activity was assayed with a mixture of 50 mM Tris-HCl buffer (pH 8.5), 5 mM $MgCl_2$, 0.5 mM NAD, 0.015 U of UDP-glucose dehydrogenase, and CFE. The reaction was started by the addition of 0.2 mM UDP-galactose.

The activity of the dTDP-rhamnose biosynthetic enzyme system was assayed in a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 0.5 mM NADPH, and CFE. Addition of 0.3 mM TDP-glucose started the reaction (Grobben *et al*., 1996).

The UDP-glucose pyrophosphorylase (EC 2.7.7.9) reverse reaction mixture contained 50 mM Tris-HCl buffer (pH 7.8), 14 mM $MgCl_2$, 0.3 mM $NADP^+$, 0.1 mM UDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 4 mM inorganic pyrophosphate (Bernstein, 1965).

The reaction mixture of the dTDP-glucose pyrophosphorylase (EC 2.7.7.24) reverse-reaction assay contained 50 mM Tris-HCl buffer (pH 7.8), 8 mM $MgCl_2$, 0.3 mM $NADP^+$, 0.1 mM TDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by the addition of 4.7 mM inorganic pyrophosphate (Bernstein, 1965).

The fructose-1,6-bisphosphatase (EC 3.1.3.11) (FBPase) assay mixture contained 50 mM glycylglycine buffer (pH 8.5), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 4 U of glucose-6-phosphate dehydrogenase, 3.5 U of phosphoglucose isomerase, and CFE. The reaction was started by adding 5 mM fructose-1,6-diphosphate (Grobben *et al*., 1996).

Sugar nucleotide analysis. CFE was prepared as described above. Immediately after preparation of the CFE, the enzymes were separated from the sugar nucleotides and other small water-soluble components by means of centrifugal filtration (5,000 x *g*, 2°C) with filter units with a nominal molecular weight limit of 10,000 (Ultrafree-MC; Millipore, Bedford, Mass.). The concentration of sugar nucleotides in the filtrates was measured by HPLC according to the method described by Harding *et al*. (1993), with a detection limit of 0.5 μmol l^{-1}. The results are the average determinations of results with bacteria harvested during three independent fermentations.

Isolation of cell-wall sugars and characterisations of EPS and cell-wall sugars. The isolation of cell-wall sugars is based on a method described by Gopal and Reilly (1995). The bacteria were grown in CDM with either 6% glucose or fructose at 30°C and pH 5.8. The bacteria were harvested at an OD_{600} of about 1.5. Lysed-cell extracts of the cultures were prepared as

described above. After ultrasonic treatment, whole cells were removed by centrifugation (3,000 x *g*, 10 min, 4°C) and the supernatant was centrifuged (20,000 x *g*, 20 min, 4°C) to harvest the cell-walls. The crude cell-wall fraction was suspended in buffer containing 140 μg of RNase and 100 μg of DNase per ml and incubated for 90 min at 37°C. The cell-walls were collected by centrifugation (20,000 x *g*, 20 min, 4°C). The obtained pellet was resuspended in buffer with 2% sodium dodecyl sulphate (SDS) and incubated at 70°C for 1 h. After centrifugation (20,000 x *g*, 20 min, 4°C), the pellet was washed three times with distilled water to remove SDS and freeze-dried, which resulted in the purified cell-wall fraction. Isolated EPS or cell-walls were hydrolysed in 4 mol l^{-1} HCl for 30 min at 100°C. Samples were dried under vacuum and dissolved in distilled water. The monomeric sugar composition after hydrolysis was determined by HPLC (van Riel and Olieman, 1991).

Activity of the *eps* promoter. *L. lactis* MG1614 harboring plasmid pNZ4040 and MG1614 harboring both pNZ4040 and the EPS plasmid pNZ4030 were used to determine the activity of the promoter of the *eps* operon (Table 2.1). Plasmid pNZ4040 contains the *eps* promoter fused to the promoterless *gus*A reporter gene, which encodes β-glucuronidase (van Kranenburg *et al*., 1997). The activity of β-glucuronidase was determined in an assay with 950 μl of GUS buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100) and 40 μl of CFE. The reaction was started by adding 10 μl of 100 mM *para*-nitro-β-D-phenyl-glucuronic acid. The increase in the A_{405} was measured at 37°C (Platteeuw *et al*., 1994). The β-glucuronidase activity was measured with CFE of MG1614 harboring pNZ4040 or of MG1614 harboring pNZ4040 and pNZ4030 to make it possible to distinguish between regulation by chromosomally or plasmid encoded factors.

Controlled overexpression of the *fbp* gene. The *E. coli fbp* gene encoding FBPase was amplified by PCR with chromosomal DNA from *E. coli* strain MC1061 (Cassadaban and Cohen, 1980) as a template and the primers 5'-CATGCCATGGCAAAAACGTTAGGTGA ATTTATTGTCG-3' and 5'-CTAGTCTAGATTACGCGTCCGGGAACTC-3'. Primer design was based on sequence data of the *E. coli fbp* gene (GenBank accession no. P09200; Hamilton *et al*., 1988), and the primers introduced flanking *Nco*I and *Xba*I restriction sites (underlined). The *fbp* gene was translationally fused to the *nisA* promoter by cloning the *Nco*I-*Xba*I digested PCR product in *Nco*I-*Xba*I digested pNZ8048, yielding pNZ4150. Plasmid pNZ4150 was transformed into *L. lactis* NZ9000 by electroporation with *E. coli* as an intermediate host. This resulted in a nisin-controlled expression system for *fbp* (Kuipers *et al*., 1998). The EPS plasmid pNZ4030 was transformed into *L. lactis* NZ9000 harboring pNZ4150 by means of electroporation.

L. lactis NZ9000 containing pNZ4150 (and pNZ4030) was grown in CDM with 0.5% sugar source at 30°C until an OD_{600} of 0.1 was reached and induced with various level of lactococcal nisin (0 to 1 ng ml^{-1}), resulting in different levels of expression of the *fbp* gene.

Results

Influence of the sugar source on EPS production. The natural-EPS-producing strain *L. lactis* subsp. *cremoris* NIZO B40 produces more EPS with glucose than with fructose as the source of sugar (Looijesteijn and Hugenholtz, 1999). Here we studied the regulation of EPS production by the carbon source by comparison of EPS-producing and non-EPS-producing cells with isogenic backgrounds, strain NZ4010 and MG1614, respectively. Strain NZ4010 was constructed by conjugal transfer of the EPS plasmid pNZ4000 of strain NIZO B40 to the EPS^- strain MG1614 (Gasson, 1983), resulting in an EPS^+ phenotype (van Kranenburg *et al*., 1997). First, EPS production of strains NZ4010 and NIZO B40 grown on glucose and fructose was determined with pH-controlled batch cultures. The amounts of EPS produced by both strains were considerably lower with fructose than with glucose as the source of sugar. Growing on glucose, the transconjugant produced less EPS than the wild type strain (Table 2.2.).

Growth of the three strains was only slightly lower with fructose as the source of sugar. The growth phase, during which most EPS is produced, was also influenced by the sugar source. During growth on glucose most of the EPS was produced during the exponential growth phase while during growth on fructose about 60% of the EPS was produced in the stationary phase (data not shown).

Table 2.2. EPS production by *L. lactis* NIZO B40, NZ4010 and MG1614 in CDM with either 6% glucose or fructose as the sugar source at 30°C and pH 5.8 and the concentration of sugar nucleotides in cell free extracts of these strains.

Strain	Sugar source	EPS (mg l^{-1})	Sugar nucleotides[a] (µmol g $protein^{-1}$)	
			UDP-glucose	UDP-galactose
NIZO B40	Glucose	460	29.9 ± 6.4	9.6 ± 2.2
	Fructose	65	ND[b]	ND[b]
NZ4010	Glucose	310	4.9 ± 2.9	2.2 ± 0.5
	Fructose	85	0.8 ± 0.7	0.4 ± 0.3
MG1614	Glucose	-	14.2 ± 1.9	4.5 ± 0.7
	Fructose	-	1.8 ± 0.6	0.6 ± 0.1

[a]) Mean ± standard deviation (n = 3).
[b]) ND, not detected, values were below the detection limit.

Influence of the sugar source on levels of expression of *eps* genes. Van Kranenburg *et al*. (1997) showed that all the *eps* genes are under the control of the *eps* promoter; hence, the activity of this promoter is a measure for the transcription levels of the *eps* genes. Plasmid pNZ4040 contains the *gus*A reporter gene, which encodes β-glucuronidase, under the control of the *eps* promoter. The activity of the *eps* promoter was determined by measuring the β-glucuronidase activity with CFE of strain MG1614 harboring pNZ4040 (and pNZ4030) grown in CDM with either glucose or fructose as the source of sugar. The activity of β-glucuronidase was

about 98 nmol mg of protein^{-1} min^{-1} for both strains grown on glucose as well as fructose, which means that the activity of the *eps* promoter does not depend on these sugar sources. From these results it can be concluded that the transcription level of the *eps* genes is not regulated by the source of sugar.

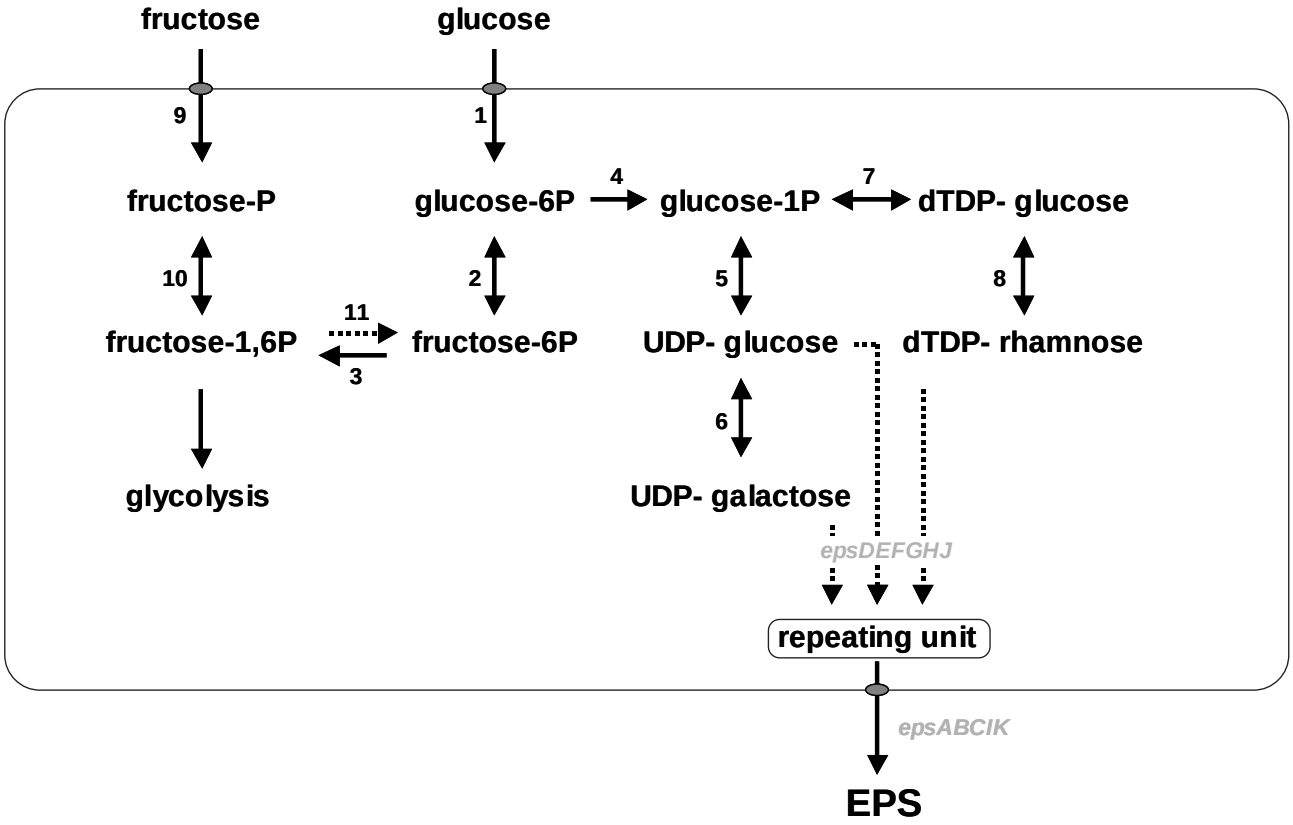

Fig. 2.1. Schematic representation of the metabolism of an EPS-producing *Lactococcus* cell grown on glucose and fructose. 1: Mannose PEP-PTS, 2: Phosphoglucose isomerase, 3: 6-Phosphofructo-kinase, 4: α-Phospho-glucomutase, 5: UDP-glucose pyrophosphorylase, 6: UDP-galactose-4-epimerase, 7: TDP-glucose pyrophosphorylase, 8: TDP-rhamnose biosynthetic enzyme system, 9: Fructose PEP-PTS, 10: 1-Phosphofructo kinase, 11: Fructose-1,6-bisphosphatase. Genes encoded on plasmid pNZ4000 are involved in the formation of the repeating unit (*epsDEFGHJ*) and in export and polymerization (*epsABCIK*) (van Kranenburg *et al*., 1997).

Influence of the sugar source on the concentration of sugar nucleotides. *L. lactis* NIZO B40 and NZ4010 produce EPS composed of glucose, galactose, rhamnose and phosphate (van Casteren *et al*., 1998). For the biosynthesis of this EPS, the activated sugar monomers UDP-glucose, UDP-galactose, and dTDP-rhamnose are necessary (Fig. 2.1). As glucose, galactose, and rhamnose are components of the cell-walls of lactococci as well, these sugar nucleotides are also necessary for cell-wall synthesis and hence for growth. The intracellular concentrations of UDP-glucose and UDP-galactose were much lower for fructose- than for glucose-grown cells (Table 2.2). The amounts of sugar nucleotides present in cells grown on glucose were higher for strain MG1614 than for strain NZ4010 (Table 2.2). All the sugar nucleotides that were found in MG1614 but not in NZ4010 were probably used for the biosynthesis of EPS. Grown on fructose the sugar nucleotide levels were only slightly lower in the EPS-producer.

This may mean that when the cells grow on fructose, only just enough sugar nucleotides are produced to fulfil the need for cell-wall biosynthesis. The affinity of the *eps* genes for the sugar nucleotides is apparently not high enough to be able to produce EPS when the concentration of these activated sugars is as low as that measured in the fructose-grown cells. EPS production on fructose took place mainly during the stationary phase, when there is no need of sugar nucleotides for growth. Cells harvested during the stationary phase contained a much higher concentration of UDP-glucose than that of cells harvested during the exponential growth phase when fructose was the substrate (data not shown).

Activities of enzymes involved in biosynthesis of sugar nucleotides. A difference in the substrate fluxes into the direction of sugar nucleotides in fructose- and glucose-grown bacteria may be caused either by a difference in the activities of the enzymes involved in their biosynthesis or by a difference in the initial sugar metabolism. The activities of enzymes involved in the biosynthesis of the EPS precursors and the initial metabolism of glucose and fructose were not influenced by the ability of cells to produce EPS (Table 2.3). During growth on fructose, the activity of 1-phosphofructokinase was significantly increased for both strains compared to that during growth on glucose. As the bacteria only need this enzyme for growth on fructose (Fig. 2.1), it is presumably induced by the presence of fructose. All the other enzymes were not significantly influenced by the source of sugar (Table 2.3).

Table 2.3. Activities of enzymes (nmol mg protein^{-1} min^{-1}) involved in initial sugar metabolism of glucose and fructose and biosynthesis of sugar nucleotides in glucose- and fructose-grown cultures of *L. lactis* NZ4010 and MG1614.

Enzyme	Glucose[a]		Fructose[a]	
	NZ4010	MG1614	NZ4010	MG1614
Mannose PTS	29 ± 13	21 ± 5	21 ± 5	28 ± 8
Phosphoglucose isomerase[b]	4318 ± 349	4085 ± 357	3415 ± 289	3148 ± 544
6-Phosphofructokinase	173 ± 44	189 ± 105	205 ± 37	210 ± 44
α-Phosphoglucomutase[b]	345 ± 41	295 ± 37	295 ± 7	290 ± 40
UDP-glucose pyrophosphorylase[b]	5.5 ± 2.2	4.2 ± 1.3	5.3 ± 1.9	4.4 ± 0.8
UDP-galactose-4-epimerase[b]	182 ± 36	143 ± 35	182 ± 7	190 ± 18
TDP-glucose pyrophosphorylase[b]	34 ± 9	29 ± 2	27 ± 6	22 ± 9
TDP-rhamnose biosynthetic system	16 ± 3	14 ± 6	14 ± 1	15 ± 1
Fructose PTS	29 ± 9	30 ± 14	22 ± 6	23 ± 9
1-Phosphofructokinase	248 ± 93	246 ± 54	658 ± 86	628 ± 134
FBPase	3.1 ± 0.8	3.5 ± 0.6	1.9 ± 0.2	2.2 ± 0.6

[a]) Mean ± standard deviation (n = at least 4).
[b]) The reversed reaction was used for measuring the activities.

Striking is the fact that the activity of FBPase was considerably lower than the activities of other enzymes involved in precursor formation and even seemed to be somewhat lower in fructose

grown cultures (Table 2.3). This enzyme is needed for the biosynthesis of sugar nucleotides when the bacteria grow on fructose but not when glucose is used as the sugar source (Fig. 2.1).

Overexpression of *fbp*. To verify if the low activity of FBPase controls the EPS production with fructose as the sugar source, the activity of this enzyme was increased by overexpression of the *fbp* gene, for which the nisin-controlled expression system was used (de Ruyter *et al*., 1996; Kuipers *et al*., 1998). The *fbp* gene from *E. coli* was fused to the *nisA* promoter in pNZ8048 (pNZ4150) and transformed into strain NZ9000. This strain contains the *nisR* and *nisK* genes, which are necessary for sensing nisin and subsequent activation of the *nisA* promoter (Kuipers *et al*., 1998), so the presence of nisin in the medium resulted in transcription of the *fbp* gene. An SDS-polyacrylamide gel of CFE of strain NZ9000 harboring pNZ4150 showed the appearance of a protein band when the bacteria were induced with nisin (data not shown). This protein band had a molecular mass of 37-kDa, which corresponds well with that of the *E. coli* FBPase (Hamilton, 1988). The intensity of the FBPase band as well as the FBPase activity increased when the concentration of nisin was increased, which proved that the expressed protein was functional.

Table 2.4. Concentration of EPS and activity of FBPase of strain *L. lactis* NZ9000 carrying pNZ4030 and pNZ4015 (FBPase) or pNZ8048 (wild-type) incubated in CDM with 0.5% fructose at 30°C and induced with different levels of nisin at an OD_{600} of 0.1.

Strain (plasmid)	Nisin (ng ml^{-1})	EPS (mg l^{-1})	(mg OD_{600}^{-1})	OD_{600}	μ^a (h^{-1})	FBPase (nmol mg $protein^{-1}$ min^{-1})
NZ9000 (pNZ8048)(wild-type)	0	1.4	1.37	1.0	0.22	6.2
	1	1.6	1.37	1.1	0.27	6.3
NZ9000 (pNZ4150)(FBPase)	0	1.3	1.21	1.1	0.23	6.7
	0.001	1.5	1.34	1.1	0.27	11.4
	0.01	2.4	1.84	1.3	0.47	78
	0.1	5.4	3.31	1.6	0.60	303
	1	8.5	4.94	1.7	0.74	628

[a]) maximum growth rate after induction, initial growth rate for both strains was 0.47 h^{-1}.

The EPS plasmid pNZ4030 was transformed into strain NZ9000 harboring pNZ4150. This new strain was grown in CDM with 0.5% fructose at 30°C under acidifying conditions. The concentration of EPS in the broth was measured at the end of the experiment. Compared to the level of EPS production in strain NZ9000 harboring pNZ4030 and the control plasmid pNZ8048, a five-fold increase in EPS production per ml was obtained with an induction level of 1 ng of nisin ml^{-1} (Table 2.4). Not only the EPS concentration but also the growth rate and the final optical density increased when the concentration of nisin in the medium was raised (Table 2.4).

Apparently, the activity of FBPase was not only limiting for EPS production but also for growth of this organism with fructose as the source of sugar. Comparable experiments with medium containing glucose as the source of sugar did not result in a change in either growth or EPS production (data not shown).

Induction of bacteria grown in medium with fructose resulted in an increase of the growth rate as well as the EPS yield (Table 2.4). To exclude the influence of the growth rate on EPS production, strain NZ9000 harboring pNZ4030 and pNZ4150 was grown in a continuous culture under leucine limitation with fructose as the source of sugar at a dilution rate of 0.2 h^{-1} with and without induction. During steady state of these cultures, the EPS concentration was about two times higher in the induced culture than in the uninduced culture. The activities of FBPase at that time were 7.5 and 105 nmol mg of $protein^{-1}$ min^{-1} in the uninduced and the induced cultures, respectively.

Influence of the sugar source on EPS and cell-wall composition. The carbon source did not influence the sugar compositions of the cell-wall polysaccharides of strains MG1614 and NZ4010 or the composition of EPS produced by NZ4010 (Table 2.5). Preliminary results indicate that the amount of cell-wall sugars is independent of the sugar source, meaning that sugar nucleotides are used preferentially for the formation of cell-wall sugars.

Table 2.5. Sugar compositions of the polysaccharides of the cell-walls of glucose- and fructose- grown *L. lactis* MG1614 and NZ4010 and of EPS produced by strain NZ4010.

Strain	Sugar source	Sugar composition (mol%)		
		Rhamnose	Galactose	Glucose
MG1614	Glucose	55.4	15.4	29.2
	Fructose	55.1	20.7	24.1
NZ4010	Glucose	51.0	17.8	31.3
	Fructose	58.0	17.2	24.8
EPS	Glucose	22.5	29.5	48.0
	Fructose	21.2	30.6	48.2

Discussion

It was shown that the EPS production by *L. lactis* NIZO B40 is far more efficient with glucose than with fructose as the source of sugar. In this chapter we describe our investigation of the possible influence of the sugar source during the different steps involved in the production of EPS by lactococci.

Enzymes leading to EPS formation can roughly be divided into four groups: enzymes responsible for the initial metabolism of a carbohydrate, enzymes involved in sugar nucleotide synthesis and interconversion, glycosyltransferases that form the repeating unit attached to the glycosyl carrier lipid, and translocases and polymerases that form the polymer. Possibilities exist for exerting control over polysaccharide synthesis at any of these four levels, and mutants lacking enzymes of any group fail to synthesize EPS (Sutherland, 1972). For *L. lactis* NIZO B40, the genes encoding the enzymes of the third and fourth groups are encoded on a plasmid and are all under the control of the *eps* promoter. The activity of this promoter was shown to be independent of the source of sugar, meaning that the transcription level of the *eps* genes is not regulated by the source of sugar. Apparently, the sugar source does not exert a specific control over EPS production by *L. lactis* but influences the polymer yield by influencing the first, unspecific steps involved in EPS biosynthesis. The second group of enzymes has been shown to control EPS synthesis in several organisms. Grobben *et al*. (1996) found that EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 is lower with fructose than with glucose as the carbon source. The activity of UDP-glucose pyrophosphorylase was higher in glucose-grown cells than in fructose-grown cells of this strain. Others also found a correlation between the activities of EPS precursor-forming enzymes and the amount of EPS produced by *Sphingomonas paucimobilis* GS1 (Ashtaputre and Shah, 1995), *Azotobacter vinelandii* (Horan *et al*., 1981), *Pseudomonas aeruginosa* (Leitão and Sá-Correia, 1995), and *E. coli* (Grant *et al*., 1970).

In case of EPS production by strain NIZO B40, no relationship between the activities of precursor-forming enzymes and the amounts of EPS produced on glucose and fructose was found, as was also the case for EPS produced by *Pseudomonas* NCIB 11264 (Williams and Wimpenny, 1980) and *Enterobacter aerogenes* (Norval and Sutherland, 1973). All the enzymes necessary for the formation of EPS precursors in strain NIZO B40 are also needed for the formation of cell-wall sugars. The genes for these enzymes are household genes and not located on the EPS plasmid (van Kranenburg *et al*., 1997). Although the activities of enzymes involved in the biosynthesis of EPS precursors were not influenced by the source of sugar, the levels of these sugar nucleotides were much lower in fructose-grown than in glucose grown *L. lactis*. Apparently, during growth on fructose, the metabolic flux in the direction of sugar nucleotides is less than during growth on glucose.

In *Lactococcus* most metabolisable sugars are transported via the PEP-PTS. Glucose is transported via the mannose PTS, which has a very low affinity for fructose. During translocation of sugars via this system, the sugars are phosphorylated at C-6. Uptake of fructose is realised mainly via the fructose PTS, resulting in fructose-1-phosphate (Benthin *et al*., 1993a). When fructose is transported via the fructose PTS, the combined actions of 1-phosphofructokinase and FBPase are required in order to form essential biomass precursors. These enzymes are not involved in the formation of biomass precursors from glucose (Benthin *et al*., 1993b). The activity of 1-phosphofructokinase was shown to be significantly higher in fructose grown cultures (Table 2.2.3), but the activity of FBPase was very low on both substrates. The low activity of this

enzyme may be responsible for the reduced production of sugar nucleotides on fructose and hence a decreased EPS production. For *Lb. bulgaricus* NCFB 2772, it was also suggested that the reduced EPS production on fructose could be caused by a more complex pathway involved in the synthesis of EPS precursors, although the levels of sugar nucleotides in this strain were only 1.5 times higher for glucose grown cultures (Grobben *et al*., 1996).

FBPase may also be involved in a 6-phosphofructokinase/FBPase catalysed ATP-consuming futile cycle in lactococci (Otto, 1984). In our continuous cultures under leucine limitation we indeed found that the concentration of lactic acid was somewhat higher and the concentration of fructose somewhat lower in the induced culture than in the uninduced culture although the biomass concentrations were equal in these cultures (data not shown).

In summary, overexpression of FBPase resulted in increased EPS production on fructose as the growth substrate. It can be concluded that the activity of this enzyme limits the amount of EPS produced by wild-type *L. lactis* on fructose. Fructose is not a common sugar source for the dairy industry, but FBPase is also required for production of biomass and EPS precursors from galactose, if it is phosphorylated at C-6 during transport via the galactose or lactose PTS (Benthin *et al*., 1993b). Furthermore, these results are also of importance when *L. lactis* is used as a cell factory for the production of EPS from sucrose or other cheap bulk materials containing fructose or galactose.

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Chapter 3

Chapter

Engineering of Carbon Distribution between Glycolysis and Exopolysaccharide Biosynthesis in *Lactococcus lactis*

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Abstract

We describe the effect of modulating activities of glucokinase, phosphofructokinase, and phosphoglucomutase on the branching point between sugar degradation and the biosynthesis of sugar nucleotides involved in the production of exopolysaccharide biosynthesis by *Lactococcus lactis*. This was realized by using described isogenic *L. lactis* mutants with reduced enzyme activities or by controlled expression of the well characterized genes for phosphoglucomutase or glucokinase from *Escherichia coli* or *Bacillus subtilis*, respectively. The role of decreased metabolic flux was studied in *L. lactis* strains with decreased phosphofructokinase activity. Neither the reduction of phosphofructokinase activity alone nor the concomitant reduction of the activity of phosphofructokinase and other enzymes encoded by the *las* operon (lactate dehydrogenase and pyruvate kinase) resulted in significant changes in the concentrations of sugar-phosphates or sugar nucleotides. In contrast, 25-fold overproduction of glucokinase resulted in seven-fold increased fructose-6P levels and two-fold reduced glucose-1P and glucose-6P levels. However, these increased sugar-phosphate concentrations did not affect the overall flux through the branching reactions leading to sugar nucleotides. Finally, 100-fold overproduction of phosphoglucomutase resulted in four-fold increased levels of both UDP-glucose and UDP-galactose. However, the increased concentrations of sugar-phosphates or sugar nucleotides did not significantly affect the production of exopolysaccharides by *L. lactis*.

Introduction

Exopolysaccharides (EPS) include a diverse range of polymers that play vital roles in a variety of biological processes. In addition, EPS have a variety of industrial applications, including their use as biothickeners in foods. Notably, EPS produced by lactic acid bacteria (LAB) contribute significantly to the structure and viscosity in fermented milk products. Furthermore, several reports indicate that they can confer health benefits to their consumer arising from their immunogenic and cholesterol-lowering properties. Although the production of EPS by LAB in milk is relatively low (below 1 g l^{-1}) in comparison with the milk sugar concentration (above 40 g l^{-1}), some EPS appear to be effective thickeners (Hess *et al.*, 1997) notably since they interact with milk proteins (van Marle and Zoon, 1995).

To study EPS biosynthesis by LAB, we have focused on *Lactococcus lactis* NIZO B40 since it produces a phosphopolysaccharide with known structure (for recent review see Boels *et al.*, 2001a). Moreover, the NIZO B40 EPS producing capacity is encoded on a 42,180-bp plasmid pNZ4000 that can be transferred to genetically well-studied model strains of *L. lactis*. (van Kranenburg *et al.*, 1997, 2000). The EPS plasmid pNZ4000 contains 14 *eps* genes involved in formation of a repeating unit by sequential addition of sugars to a membrane-anchored lipid-carrier, and export en polymerization of these repeating units. Upon its transfer to model strains, the NIZO B40 phosphopolysaccharide is produced that contains glucose, galactose and rhamnose moieties. Therefore, their production require the sugar nucleotides UDP-glucose, UDP-galactose and dTDP-rhamnose, which are formed by enzymes encoded on the chromosome (Fig. 3.1). The central intermediate, glucose-1-phosphate (glucose-1P), is converted to dTDP-rhamnose by the sequential activities of the *rfbACBD* gene products (Boels *et al.*, 2002). GalU activity catalyzes the conversion of glucose-1P into UDP-glucose, which is subsequently converted into UDP-galactose by the GalE activity (Boels *et al.*, 2001b). Presently, all the genes that encode the enzymes involved in the biosynthesis of these sugar nucleotides from glucose-1P (*galU*, *galE*, and *rfbACBD*; Fig. 3.1) have been cloned from *L. lactis* MG1363 and their role in controlling the sugar nucleotide levels have been investigated (Boels *et al.*, 2001b, 2002).

Insight in the biosynthesis of EPS is crucial for the exploitation of microorganisms for the production of EPS with industrial or medical importance. It is relevant for the design of metabolic engineering strategies that aim at increased fluxes to EPS production to include control factors in sugar degradation and EPS formation pathways (de Vos, 1996). In *L. lactis* used in dairy fermentations, sugar degradation starts with lactose uptake via a phosphotransferase system, which yields, after hydrolysis, galactose-6P and glucose moieties. Subsequently, the galactose-6P moiety can be catabolized completely via the tagatose pathway and glycolysis for the generation of biomass and energy, while the glucose moiety could be used for EPS production (de Vos, 1996). Uncoupling of lactose-derived glucose and galactose metabolism has previously

been established in a *L. lactis* mutant impaired in glucokinase and glucose phosphotransferase system activity that accumulated glucose (Thompson *et al.*, 1985). Assuming that the linkage between glycolysis and EPS formation occurs at the branching point starting from the glycolysis intermediate glucose-6P, it is tempting to speculate that it is possible to engineer EPS overproduction by increasing the pool of this sugar-phosphate.

The availability of glucose-6P may be affected by modulation of glycolytic activity. It has been reported that the lactococcal glycolytic flux can be affected by the activity of the global catabolite control protein, CcpA (Luesink *et al.*, 1998). This protein acts as an activator of transcription of the *las* operon (Llanos *et al.*, 1993) encoding the glycolytic enzymes phosphofructokinase (Pfk), pyruvate kinase (Pyk), and the lactate-forming enzyme lactate dehydrogenase (Ldh). Furthermore, modulation of the glucose-6P pool could potentially be achieved by engineering the enzyme activities that are involved in the formation and degradation of this intermediate, such as glucokinase (Glk) and Pfk activity, respectively (Fig. 3.1).

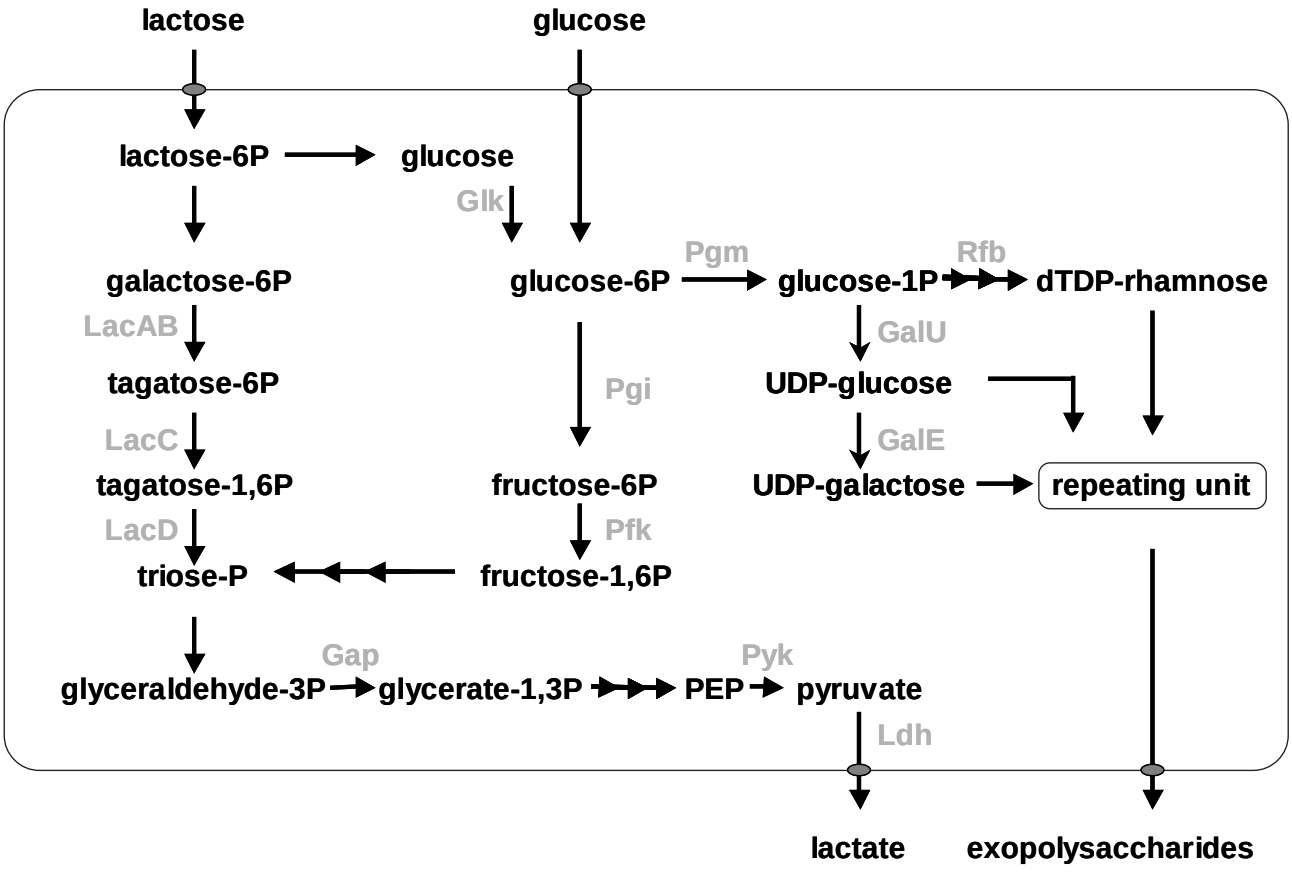

Fig. 3.1. Schematic representation of pathways involved in sugar fermentation via glycolysis to lactate and/or other acids and biosynthesis of exopolysaccharides (EPS) in *L. lactis*. Glucose and lactose are transported via phosphotransferase systems. Enzymes involved: Glk, glucokinase; Pgi, phosphogluco isomerase; Pfk, phophofructokinase; Gap, glyceraldehyde 3-phosphate dehydrogenase; LacABCD, tagatose-6-phosphate pathway; Pyk, pyruvate kinase, Ldh; lactate dehydrogenase; Pgm, phosphoglucomutase; GalU, UDP-glucose pyrophosphorylase; GalE, UDP-galactose epimerase; Rfb: dTDP-rhamnose biosynthetic system consisting of RfbA, glucose-1P thymidylyltransferase; RfbB, dTDP-glucose-4,6 dehydratase; RfbC, dTDP-4-keto-6-deoxy-*D*-glucose-3,5 epimerase; and RfbD, dTDP-4-keto-*L*-rhamnose reductase.

Another possible key-step in sugar nucleotide biosynthesis is the inter-conversion of the glycolysis intermediate glucose-6P, and glucose-1P, which can be regarded as the central precursor in sugar nucleotide biosynthesis (Sjöberg and Hahn-Hägerdal, 1989), which is performed by phosphoglucomutase (Pgm, Fig. 3.1). *L. lactis* contains two distinct forms of Pgm, one specific for α-glucose-6P (i.e. α-Pgm) and the other specific for β-glucose-6P (i.e. β-Pgm)(Qian *et al.*, 1994). Since the phosphotransferase sugar uptake or the glucokinase (Glk)-mediated phosphorylation of glucose yields α-glucose-6P (Ramos *et al.*, 2001), α-Pgm could be a key-enzyme in sugar nucleotide biosynthesis. However, presently only the gene encoding β-Pgm has been identified in *L. lactis* (Qian *et al.*, 1997).

We studied the effect of modulation of enzyme activities at the branching point between sugar degradation and sugar nucleotide and EPS biosynthesis. By influencing expression of the corresponding genes, we evaluated the role of CcpA (Luesink *et al.*, 1998), Pfk activity (Andersen *et al.*, 2001), and controlled overproduction of heterologous Pgm and Glk in biosynthesis of NIZO B40 EPS in the plasmid-free model strain *L. lactis* MG1363. It could be established that the overproduction of *B. subtilis* Glk and *E. coli* Pgm significantly increased sugar-phosphates and sugar nucleotides, respectively. However, this did not affect the NIZO B40 EPS production level. Furthermore, although the global effect of the *ccpA* mutant strain did not show any changes in the levels of the sugar nucleotides, it produced significantly less EPS than the wild-type strain.

Materials and methods

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 3.1. *E.coli* MC1061 (Casadaban and Cohen, 1980), which was used as a host in cloning experiments, was grown with aeration in TY-broth at 37°C. *L. lactis* was grown without aeration at 30°C in a chemically defined medium (CDM) (Looijesteijn and Hugenholtz, 1999) or in M17 broth (Merck, Darmstadt, Germany), supplemented with 0.5% (wt vol^{-1}) glucose or lactose. When appropriate, the media contained chloramphenicol (10 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (2 μg ml^{-1}) or ampicillin (100 μg ml^{-1}). To analyze the effect of gene overexpression the nisin-controlled expression system (NICE) was used (de Ruyter *et al.*, 1996a, Kuipers *et al.*, 1998). For enzyme activity analysis *L. lactis* cells were grown till an optical density (OD) at 600 nm of approximately 0.5 and for EPS analysis the cells were grown till an OD of approximately 0.1. Subsequently the culture was split into two cultures. One ng ml^{-1} of nisin was added to one of the two cultures, and both cultures were grown for an additional 2 h to 24 h.

Table 3.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
L. lactis		
MG1363		(Gasson, 1983)
MG5267		(van Rooijen *et al.*, 1992)
NZ3800	MG5267 *pepN::nisRK*	This work
NZ9870	MG1363 *ccpA*::*ery*	(Luesink *et al.*, 1998)
NZ9000	MG1363 *pepN::nisRK*	(Kuipers *et al*., 1998)
HWA217		(Andersen *et al*., 2001)
E. coli		
MC1061		(Casadaban and Cohen, 1980)
B. subtilis		
ATCC 6633		ATCC[b] collection
Plasmids		
pMG820	Lac^+	(Maeda and Gasson, 1986)
pNZ4123	Cm^r, pNZ8048 derivative containing a functional *E. coli pgmU* gene	This work
pNZ4124	Cm^r, pNZ8048 derivative containing a functional *B. subtilis glk* gene	This work
pNZ4030	Ery^r, Eps^+	(van Kranenburg *et al.*, 1997)
pNZ4130	Tet^r, Eps^+	(Boels *et al.*, 2001b)
pNZ9573	Ery^r, non replicative lactococcal plasmid *pepN::nisRK*	(de Ruyter *et al.*, 1996b)
pNZ8048	Cm^r, inducible expression vector, carrying the *nisA* promoter	(Kuipers *et al*., 1998)

[a]) Lac^+, Lactose-fermenting phenotype; Eps^+, EPS-producing phenotype; Cm^r, chloramphenicol resistant, Ery^r, erythromycin resistant; Tet^r, tetracycline resistant.
[b]) ATCC, American Type Culture Collection.

DNA manipulations and DNA sequence analysis. Small scale isolation of *E. coli* plasmid and chromosomal DNA and standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Isolation and transformation of *L. lactis* DNA were performed as previously described (de Vos *et al.*, 1989). Isolation of chromosomal DNA of *B. subtilis* was performed as described by Bron (1990).

Automatic double-stranded DNA sequence analysis was performed on both strains with an ALF*red* DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands). Sequence reactions were performed with an Autoread kit, were initiated by using Cy5-labeled universal and reverse primers, and were continued with synthetic primers purchased from Pharmacia Biotech in combination with fluorescein-15-dATP by following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed by using the PC/GENE program, version 6.70 (Intelli-Genetics).

Construction of strains and plasmids. To make the *L. lactis* MG1363 derivative MG5267 a suitable host for the use of the nisin inducible expression (NICE) system, the lactococcal *nisRK* genes were introduced into the *pepN* locus as described by de Ruyter *et al.* (1996b). The resulting strain, designated NZ3800, contains the *nisRK* genes under control of their own promoter integrated in the *pepN* locus. The expected genetic configuration of the *pepN::nisRK* locus was confirmed by PCR analysis.

The lactose-fermenting capacity was introduced into strain *L. lactis* HWA217 (Andersen *et al.*, 2001) by introduction of the mini-lactose plasmid pMG820 into this strain (Maeda and Gasson, 1986). Subsequently, the EPS producing capacity was introduced into the resulting strain by transformation of plasmid pNZ4030, an erythromycin-resistant-derivative of the NIZO B40 Eps-plasmid, pNZ4000 (van Kranenburg *et al.*, 1997).

The EPS producing capacity was introduced into *L. lactis* CcpA mutant strain NZ9870 (Luesink *et al.*, 1998) by transformation of plasmid pNZ4130 (Boels *et al.*, 2001b), a tetracycline-resistant-derivative of pNZ4000.

The *E. coli* α-phosphoglucomutase (*pgmU*) gene was amplified by PCR using *Tth* polymerase and chromosomal DNA of *E. coli* MC1061 (Casadaban and Cohen, 1980) as template DNA with the primers 5'-CATGCCATGGCAATCCACAATCGTGCAG -3' and 5'-CTAGTCTAGATTACGCGTTTTTCAGAACTTCGC -3'. The 1.64-kb PCR product generated was cloned into pNZ8048 (Kuipers *et al.*, 1998) using the *Nco*I and *Xba*I restriction sites that were introduced by the primers used (underlined), yielding the *pgm* overexpression plasmid pNZ4123.

The *B. subtilis* glucokinase (*glk*) gene was amplified by PCR using *Tth* polymerase and chromosomal DNA of *B. subtilis* ATCC 6633 (Gordon *et al.*, 1973) as template DNA with the primers 5'-CATGCCATGGACGAGATATGGTTTGCG -3' and 5'-CTAGTCTAGATTAACAATTTTGATGTTTCA -3'. The 0.98-kb PCR product generated was cloned into pNZ8048 (Kuipers *et al.*, 1998) using the *Nco*I and *Xba*I restriction sites that were introduced by the primers used (underlined), yielding the *glk* overexpression plasmid pNZ4124.

The plasmid pNZ4123 or pNZ4124 was introduced into *L. lactis* strain NZ3800 by transformation. Subsequently, the EPS producing capacity was introduced in *L. lactis* NZ3800 harboring pNZ4123 or pNZ4124 by electroporation of plasmid pNZ4030 (van Kranenburg *et al.*, 1997).

Preparation of cell-free extracts and protein analysis. Lactococcal cells (50 ml) were harvested by centrifugation (3,500 x g, 10 min, 4°C) and cell pellets were resuspended in 1 ml of 20 mM sodium-phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. These suspensions were mechanically disrupted by bead-beating in the presence of Zirconium beads (van der Meer *et al.,* 1993) and cell debris was removed by centrifugation (3,500 x g, 10 min, 4°C), resulting in the cell-free extract (CFE). The protein content of the CFE was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

For protein analysis lactococcal CFE was mixed with an equal amount of two-fold concentrated Laemmli buffer and, after boiling, 10 μg of each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Enzyme assays. Enzyme reactions were performed at 30°C in a total volume of 1-ml containing various amounts of freshly prepared CFE. The formation of NADH or NADPH was determined by measuring the change of the absorbence at 340 nm. Given values are the means of at least two independent measurements. The blank contained the reaction buffer, cofactors and the substrate, but lacked the CFE.

The glucokinase (Glk; EC 2.7.1.2) reaction assay contained 100 mM triethanolamine-hydrochloride buffer (pH 7.8), 5 mM $MgCl_2$, 1 mM $NADP^+$, 4 U of glucose-6P dehydrogenase, 2 mM ATP and CFE. The reaction was initiated by the addition of 10 mM glucose (Petit *et al.* 1991).

The α-phosphoglucomutase (Pgm; E.C. 2.7.5.1) reaction assay contained 50 mM triethanolamine buffer (pH 7.2), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 50 M glucose-1,6P, 4 U of glucose-6P dehydrogenase, and CFE. The reaction was initiated by the addition of 1.4 mM α-glucose-1P (Qian *et al.*, 1994).

Estimation of intracellular metabolites. The intracellular metabolites glucose-6P, glucose-1P and fructose-6P were fixed by rapid inactivation of cell metabolism. Therefore, four ml of cell culture in the logarithmic growth phase were mixed with an equal amount of cold (-80°C) methanol. Methanol was removed by evaporation in a heating block and cell metabolites were measured by coupling appropriate enzyme assays with fluorimetric determination of NADPH as described by Garrigues *et al.* (1997).

Sugar nucleotide and EPS analysis. Sugar nucleotides were separated from CFE, and individual sugar nucleotide contents were determined by high-performance liquid chromatography as previously described by Looijesteijn *et al.* (1999). The values reported are the average of at least two independent determinations. EPS was isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (1999).

Results

Glycolytic flux modulation. By modulation of the glycolytic flux we investigated the possibilities to increase the pool of glucose-6P, the level of sugar nucleotides, and subsequently the level of EPS production. In an initial approach we evaluated the effect of reduced glycolytic flux on EPS

biosynthesis by analyzing NIZO B40 EPS biosynthesis in *L. lactis ccpA* disruption strain NZ9870. In this strain the transcription level of the *las* operon genes (*pfk*, *pyk* and *ldh*), is four-fold reduced compared to that of its parental strain (Leusink *et al.*, 1998). Moreover, the activity of other enzymes may be affected as well since CcpA is a global control protein. The *ccpA*-disruption strain NZ9870 showed reduced growth rate, reduced glycolytic flux and a switch from homolactic to mixed-acid fermentation (Luesink *et al.*, 1998). In this strain, the levels of the first two glycolytic-intermediates glucose-6P and fructose-6P appeared to be two- and seven-fold increased relative to the levels measured in the wild-type strain (Table 3.2). However, neither the level of glucose-1P (Table 3.2) nor the levels of sugar nucleotides UDP-glucose and UDP-galactose and dTDP-rhamnose (data not shown) were influenced by the changed level of glucose-6P. Consequently, no effect of the *ccpA* mutation on EPS production level was expected. However, EPS production in this strain appeared two-fold reduced relative to the wild-type strain (Table 3.3), while this could suggest that CcpA exerts control on EPS production at the level of EPS machinery itself. It is also likely that, the overall reduced metabolic rate in the *ccpA* mutant results in reduced energy availability, which could negatively influence EPS production.

Table 3.2. Enzyme activities, sugar nucleotide, and glucose-6P, fructose-6P (Frc-6P), glucose-1P concentrations in *L. lactis* subsp. *cremoris* MG1363 derivatives grown in M17 media with glucose (Glc) or lactose (Lac) as sole carbon source.

Strain (plasmid)	Sugar source	Nisin (ng ml^{-1})	Relevant enzyme activity[a] (mU protein^{-1})	Sugar-phosphates[b] (μM mg of protein^{-1})		
				Glc-6P	Frc-6P	Glc-1P
NZ3800	Glc	0	ND[c]	23	9.7	39
MG1363 (pMG820)	Lac	0	ND	31	44	37
NZ9870	Glc	0	ND	47	68	46
HWA217 (pMG820)	Lac	0	ND	32	32	8.0
NZ3800 (pNZ4123)	Lac	0	222 ± 42[d]	30	25	35
	Lac	1	24767 ± 506[d]	30	33	33
NZ3800 (pNZ4124)	Lac	0	352 ± 51[e]	5.2	74	10
	Lac	1	9482 ± 93[e]	13	290	10

[a]) The values are based on at least two independent experiments.
[b]) The values are based on one single experiment.
[c]) ND, not determined.
[d]) Pgm specific activity.
[e]) Glk specific activity.

In a more controlled approach to decrease glycolytic flux we evaluated the role of the Pfk enzyme in EPS biosynthesis by analyzing NIZO B40 EPS biosynthesis in *L. lactis* strain HWA217 (Andersen *et al.*, 2001). In this strain the *las* promoter, which drives Pfk, Pyk and Ldh activity, has been substituted by a synthetic promoter resulting in two-fold reduction of Pfk activity, while the activity levels of Pyk and Ldh remain close to that observed in the wild-type (Andersen *et al.*,

2001). When fructose was used as a substrate the growth rate of the mutant was comparable to that of MG1363 when grown on fructose, but in medium containing glucose the growth rate was almost two-fold reduced (data not shown). These results confirm previous results described for this strain (Andersen *et al.*, 2001). To be able to grow the *L. lactis* MG1363 and its derivative HWA217 on a medium with lactose as the sole carbon source, the lactose-fermenting capacity was introduced into these strains by transformation of the lactose mini-plasmid pMG820 (Maeda and Gasson, 1986). The sugar-phosphate levels in glucose-grown cells of strain MG1363 harboring pMG820 were significantly higher compared to those of lactose-grown cells of strain NZ3800, indicating that the mode of entrance of glucose into glycolysis is different. In contrast to the *ccpA* mutant the intracellular levels of glucose-6P and fructose-6P were not increased in strain HWA217 compared to those observed in strain MG1363 (Table 3.2). These observations are in contrast to those that were previously reported for glucose grown cultures of strain HWA217 (Andersen *et al.*, 2001) and appears to suggest that the effect of Pfk activity levels in these primary glycolytic intermediates depends on the mode of entrance of glucose into glycolysis. However, the level of glucose-1P appeared to be three-fold decreased in strain HWA217, which is an observation that remains unexplained. In this respect, it should be noted that the values given are based on a single, indicative experiment.

Table 3.3. EPS production *L. lactis* MG1363 derivatives harboring pNZ4030 grown in CDM with glucose or lactose as sole carbon source.

Strain (plasmid)[a]	C-source	EPS[b] (mg $l^{-1} * OD_{600}^{-1}$)
MG1363	Glucose	36 ± 1
MG1363 (pMG820)	Lactose	38 ± 1
NZ9870	Glucose	16 ± 2
HWA217	Glucose	43 ± 1
HWA217 (pMG820)	Lactose	39 ± 2

[a]) Strain NZ9870 harbors pN4130, other strains harbor pNZ4030.
[b]) The values are averages based on at least two independent experiments.

No difference in EPS production was observed when cells were grown in media with glucose or lactose as carbon source (Table 3.3), suggesting that a reduced Pfk activity level does not affect EPS biosynthesis.

To evaluate the effect of stimulated phosphorylated-sugar influx in glycolysis on EPS biosynthesis in *L. lactis*, we studied the effect of controlled *glk* overexpression using the NICE system (de Ruyter *et al.*, 1996a; Kuipers *et al.*, 1998). Since the sequence of the *L. lactis glk* gene was not available we used a heterologous approach as a proof of principle. Therefore, we cloned the *B. subtilis* ATCC 6633 *glk* gene under control of the lactococcal *nisA* promoter and the resulting plasmid (pNZ4124) was introduced in strain NZ3800. Strain NZ3800 harboring

pNZ4124 was grown under inducing and non-inducing conditions, and CFE of the cultures were prepared and analyzed by SDS-PAGE (Fig. 3.2). Growth in the presence of nisin resulted in the appearance of an additional protein band with an apparent molecular mass of approximately 34-kDa, which is the expected size of Glk. Moreover, more than 25-fold increased Glk specific activity was obtained with CFE of the induced cultures compared to that in the control cultures (Table 3.2). Functional overexpression of *glk* resulted in an almost seven-fold increased fructose-6P level compared to that of the wild-type strain (Table 3.2). Remarkably, both the level of glucose-6P as well as the level of glucose-1P were reduced, suggesting that a substrate activation may take place of phosphogluco-isomerase (Pgi) activity. Pgm apparently maintains a constant ratio between glucose-6P and glucose-1P, which is independent of the glucose-6P concentration. If so, the low glucose-1P level observed in the strain HWA217, can not easily explained, unless in this strain there are other pleiotropic effects. The increased Glk activity did not affect the sugar nucleotide levels (data not shown) nor the NIZO B40 EPS production level (Table 3.3), suggesting that Glk does not play an important role in control of sugar nucleotide and EPS biosynthesis in *L. lactis*.

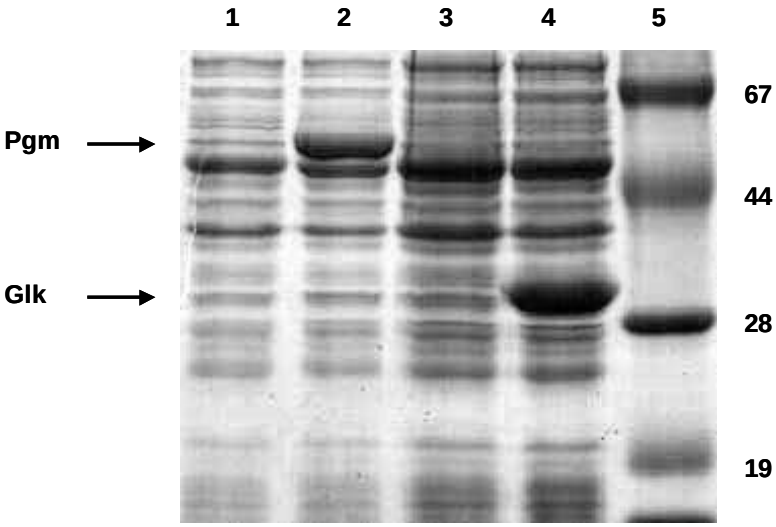

Fig. 3.2. Coomassie blue-stained gel after SDS-PAGE of CFE of *L. lactis* NZ3800 harboring pNZ4123 (lane 1 and 2) or pNZ4124 (lane 3 and 4) grown in the absence (lane 1 and 3) and in the presence (lane 2 and 4) of nisin. Lane 5 contained a set of protein standards with molecular masses [in kDa] indicated on the right. Additional bands resulting from nisin induction and representing Pgm protein (lane 2) and Glk protein (lane 4) are indicated.

Branching point modulation. To evaluate the role of increased Pgm activity in UDP-sugar formation and EPS biosynthesis in *L. lactis*, we studied the effect of controlled *pgm* overexpression by using the NICE system (de Ruyter *et al.*, 1996a; Kuipers *et al.*, 1998). Similar to *glk* overexpression we used a heterologous approach to generate a proof of principle. Therefore, we cloned the *E. coli* K12 *pgmU* gene under control of the lactococcal *nisA* promoter (pNZ4123). Strain NZ3800 harboring pNZ4123 was grown under inducing and non-inducing conditions, and CFE of the cultures were analyzed by SDS-PAGE (Fig. 3.2). Nisin induction resulted in the appearance of a protein band with an apparent molecular mass of approximately 58-kDa, corresponding to the expected size of PgmU. In analogy, a 100-fold increased Pgm specific activity was measured in the CFE of the induced cultures (Table 3.2). These results demonstrate the functional overexpression of the *pgmU* gene. Despite this high level Pgm production no differences in growth rates between nisin-induced NZ3800 harboring pNZ4123 and the uninduced strain were observed (data not shown).

To study the effect of *pgmU* overexpression on sugar nucleotide levels, the concentrations of UDP-glucose and UDP-galactose were determined of NZ3800 harboring pNZ4123 grown in the presence of nisin. Overexpression of *pgmU* resulted in a four-fold increase in the levels of both UDP-glucose and UDP-galactose (Fig. 3.3), while dTDP-rhamnose levels remained the same (data not shown). These results indicate that the activity level of Pgm to some extent controls UDP-glucose and UDP-galactose biosynthesis in *L. lactis*. In contrast, the increased level of PgmU activity did not have a significant effect on the level of EPS production when cells were grown in CDM with glucose or lactose as a sole carbon source (Table 3.4). These results indicate that the improved UDP-glucose and UDP-galactose levels in NZ3800 harboring pNZ4123 have no effect on the EPS biosynthesis efficiency in this strain.

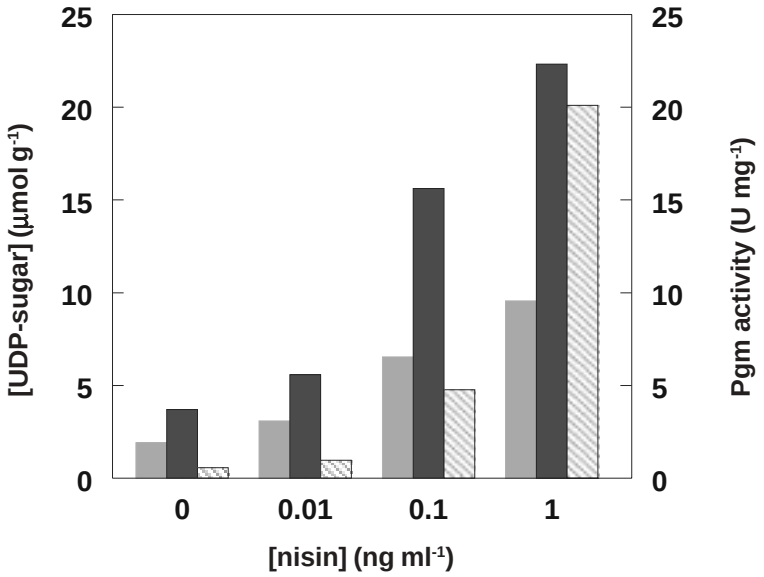

Fig. 3.3. UDP-galactose (grey bar), UDP-glucose (dark gray bar) levels, and Pgm activity (dashed bar) of *L. lactis* NZ9000 harboring pNZ4123 grown in the presence of different levels of nisin.

Table 3.4. EPS production *L. lactis* NZ9000 derivatives harboring pNZ4030 grown in CDM with glucose or lactose as sole carbon source.

Strain (plasmid)[a]	Nisin (ng ml^{-1})	EPS[b] (mg l * OD_{600}^{-1}) Glucose	Lactose
NZ3800 (pN8048)	0	46 ± 1	39 ± 1
NZ3800 (pNZ4123)	0	42 ± 1	38 ± 1
	1	49 ± 1	39 ± 1
NZ3800 (pNZ4124)	0	46 ± 1	37 ± 3
	1	50 ± 1	39 ± 1

[a]) All strains harbor pNZ4030.
[b]) The values are averages based on at least two independent experiments.

Discussion

The biosynthesis of EPS involves a large number of household enzymes required for the production of EPS precursors, and EPS specific enzymes that are encoded in *eps* gene clusters. In this study we focused on the regulation of EPS production by three household enzymes: glucokinase, phosphoglucomutase and phosphofructokinase, all acting at the branching point between glycolysis and EPS precursor biosynthesis (Fig. 3.1), which is a potential bottleneck in sugar nucleotide and subsequent EPS biosynthesis. In this respect, increasing the intracellular glucose-6P pool, which is the central branching point intermediate, might push metabolism towards sugar nucleotide biosynthesis and EPS production. It has been reported that increased glucose-6P levels could be toxic at enhanced levels (Fraenkel, 1968), although accumulation of lactose-phosphate was tolerated by *L. lactis* deficient in LacG activity (Crow and Thomas, 1984). Therefore, it is feasible that concomitant enhancement of sugar anabolism leading to the increased production of sugar nucleotides and subsequently EPS might relieve glucose-6P toxic effects by decreasing the pool of glucose-6P in the cell and could lead to enhanced EPS production.

In an initial approach we evaluated the effect of reduced glycolytic flux on EPS biosynthesis by analyzing a *ccpA* mutant. Such mutants are known to be reduced in glycolytic flux and the *L. lactis ccpA* mutant has a four-fold reduced transcription level of the *las* operon, containing the *pfk*, *pyk* and *ldh* genes (Luesink *et al.*, 1998). However, it is also known that CcpA is a global regulator and has pleiotropic effects. Remarkably, the concentrations as well as the ratio of fructose-6P and glucose-6P were increased in the *ccpA* mutant strain. These results establish that overall reduction of the enzyme activities encoded by the *las* operon leads to accumulation of intermediates high-up in the glycolytic pathway. Although the *ccpA* mutation led to an increase of the pool of glucose-6P, the overall flux towards EPS production was approximately two-fold reduced compared to that in the wild-type level. Since no catabolite control elements were found in any of the sequences of genes involved in sugar nucleotide or EPS biosynthesis (data not shown), it seems unlikely that CcpA regulates expression of any of these genes directly. It might be possible that the overall reduced metabolic rate in the *ccpA* mutant resulted in reduced energy availability for EPS biosynthesis.

A more targeted approach was taken to evaluate whether decreased glycolytic flux controls EPS production by analyzing a *L. lactis* mutant strain with reduced Pfk activity (Andersen *et al.*, 2001). It has been reported that glucose-6P and fructose-6P accumulate in this mutant when it is grown on glucose (Andersen *et al.*, 2001). We were unable to confirm these observations in cells grown on lactose. While unexplained effects in sugar-phosphate concentration were observed, UDP-sugar levels and NIZO B40 EPS production were not influenced in this *pfk* mutant. These results indicate that Pfk activity does not exert direct control on EPS biosynthesis in *L. lactis*.

In another approach to modulate glycolytic flux, we aimed at glucose-6P accumulation as a result of increased Glk activity. Heterologous overexpression of *B. subtilis glk* in *L. lactis* resulted in more than 25-fold increase in enzyme activity level. Remarkably, the level of glucose-6P and glucose-1P were decreased, while the level of fructose-6P was almost seven-fold increased. These observations suggest that phosphogluco-isomerase (Pgi) activity is subject to substrate glucose-6P activation. However, the increased Glk activity level did not result in more EPS precursors and NIZO B40 EPS. These results imply that the level of Glk enzyme activity does not control NIZO B40 EPS production levels.

It has been shown that mutations in the *pgm* gene affect the morphology of *E. coli* (Lu and Kleckner, 1994) and affected polysaccharide composition of *Pseudomonas aeruginosa* (Coyne *et al.*, 1994; Ye *et al.*, 1994) and *Streptococcus pneumoniae* (Hardy *et al.*, 2000, 2001). However, in *L. lactis* the α-Pgm is likely to be essential for sugar nucleotide formation and thus essential for growth and viability of this species (Ramos *et al.*, 2001). To investigate the role of Pgm at the branching point between sugar catabolism and anabolism in *L. lactis*, we evaluated the effect of increased levels of Pgm activity. Heterologous overexpression of *E. coli pgmU* in *L. lactis* resulted in a more than 100-fold increase in enzyme activity level and a four-fold increases in UDP-glucose and UDP-galactose levels. These results show that the level of PgmU enzyme activity controls the level of production of UDP-glucose and UDP-galactose in wild-type cells. However, the sugar-phosphate analysis of the Glk overproduction strain suggest that the endogeneous Pgm activity maintains a stable glucose-6P to glucose-1P ratio. These results suggest that Pgm overproduction will have no effect on sugar nucleotide synthesis. Therefore, It is likely that the increased sugar nucleotide levels are the result of favorable kinetic parameters (K_m, V_{max}) of the *E. coli* PgmU. However, increased Pgm activity did not affect NIZO B40 EPS production (Table 3.3). In analogy, GalU overproduction led to the same result (Boels *et al.*, 2001b). Similar findings were reported for *Sphingomomas* in which six-fold increase of Pgm activity did not affect EPS production (Thorne *et al.*, 2000). Contrasting results were reported for *Streptococcus thermophilus* strain LY03. Degeest and De Vuyst (2000) showed a linear relationship between Pgm activity and EPS production, while Levander and Rådström (2001) reported that overexpression of *pgm* in the same strain did not change EPS production. It is suggested that precursors for EPS biosynthesis in a galactose-fermenting strain of *S. thermophilus* originate from fermentation of the galactose moiety of lactose, hereby circumventing the need for a functional Pgm in these cells (Levander and Rådström, 2001). Interestingly, when *pgm* was overexpressed in combination with *galU* the EPS yield was shown to increase two-fold in this strain (Levander *et al.*, 2002).

Evaluation of the EPS biosynthesis model described here allowed us to assess the role of the Glk and Pfk in *L. lactis* by modulating their enzyme activity levels. Although early glycolytic intermediates like glucose-6P and fructose-6P accumulated in some cases, these modulations did not affect the overall flux through the branching reactions leading to sugar nucleotide and eventually EPS. In contrast, modulation of Pgm activity resulted in significantly increased internal

levels of both UDP-glucose and UDP-galactose. However, Pgm overproduction did not result in a significant increase of NIZO B40 EPS. Remarkably, a *ccpA* mutant strain produced only half the amount of EPS compared to wild-type cells, which might be caused by the overall reduced metabolic rate in the *ccpA* mutant resulting in reduced energy availability for EPS biosynthesis. The results presented here provide insight in enzymatic control factors that determine the flux distribution between catabolic and anabolic reactions involved in conversion of the central glycolytic intermediate glucose-6P. Such insight is essential for the design of metabolic engineering strategies that aim at resolving bottlenecks in EPS biosynthesis in *L. lactis*.

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Chapter 4

Chapter

Functional Analysis of the *Lactococcus lactis galU* and *galE* Genes and their Impact on Sugar Nucleotide and Exopolysaccharide Biosynthesis

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Abstract

We studied the UDP-glucose pyrophosphorylase (*galU*) and UDP-galactose epimerase (*galE*) genes of *Lactococcus lactis* MG1363 to investigate their involvement in biosynthesis of UDP-glucose and UDP-galactose which are precursors of glucose- and galactose containing exopolysaccharides (EPS) in *L. lactis*. The lactococcal *galU* gene was identified by a PCR approach using degenerate primers and was found by Northern blot analysis to be transcribed in a monocistronic RNA. The *L. lactis galU* gene could complement an *Escherichia coli galU* mutant, and overexpression of this gene in *L. lactis* under control of the inducible *nisA* promoter resulted in a twenty-fold increased in GalU activity. Remarkably, this resulted in approximately eight-fold increases in the levels of both UDP-glucose and UDP-galactose. This indicated that the endogenous GalE activity is not limiting and that the GalU activity level in wild-type cells controls the biosynthesis of intracellular UDP-glucose and UDP-galactose. The increased GalU activity did not significantly increase NIZO B40 EPS production. Disruption of the *galE* gene resulted in poor growth, undetectable intracellular levels of UDP-galactose, and elimination of EPS production in strain NIZO B40 when cells were grown in media with glucose as the sole carbon source. Addition of galactose restored wild-type growth in the *galE* disruption mutant, while the level of EPS production was approximately one-half the wild-type level.

Introduction

Lactic acid bacteria are widely used for production of fermented foods, where they are responsible not only for production of lactic acid as a food preservative but also for generation of flavor and texture. Several lactic acid bacteria produce exopolysaccharides (EPS). These EPS contribute to the rheological properties and texture of fermented products and are therefore of interest for food applications as natural biothickeners (Sutherland, 1998). Moreover, it has been suggested that EPS may confer health benefits to the consumer, and mouse model studies have indicated that EPS may have immunostimulatory (Hosono *et al.*, 1997), antitumoral (Kitzawa *et al.*, 1991), or cholesterol-lowering activity (Nakajima *et al.*, 1992a).

Microbial polysaccharides can be present as constituents of cell-walls, as parts of lipopolysaccharides (LPS), often referred to as O-antigens**,** or as capsular polysaccharides (CPS) associated with the cell surface, or they can be secreted as EPS in the environment of the cell. Detailed knowledge concerning microbial polysaccharide biosynthesis and the biophysical characteristics of these molecules accumulated over the years (Sutherland, 1998). Different classes of EPS can be distinguished on basis of their biosynthesis mechanisms and the precursors required (Sutherland, 1993). They can be synthesized either extracellularly from exogenous substrates or intracellularly from sugar nucleotide precursors. Many EPS contain repeating units, the biosynthesis of which involves glycosyltransferases that sequentially link sugars from intracellular nucleotide sugars to a lipid carrier. This mechanism resembles the mechanism of the production of O-antigens and several types of CPS (Roberts, 1996). It is closely related to the mechanism of biosynthesis of cell envelope components like peptidoglycan (Park, 1987) and teichoic acid (Archibald *et al.*, 1993), since in all of these mechanisms assembly takes place on a common lipid carrier that is situated in the cell membrane.

Genes involved in EPS biosynthesis are organized in gene clusters which appear to be highly conserved. The gene clusters that direct EPS biosynthesis in *Lactococcus lactis* NIZO B35, NIZO B40, and NIZO B891 (van Kranenburg *et al.*, 1997, 1999a) and *Streptococcus thermophilus* Sfi6 (Stingele *et al.*, 1996), are comparable to the gene clusters in *Streptococcus pneumoniae* (Morona *et al.*, 1997) and *Streptococcus agalactiae* (Yamamoto *et al.*, 1999) involved in CPS biosynthesis (van Kranenburg *et al.*, 1999b; Stingele *et al.*, 1996). These gene clusters encode enzymes which are involved in formation of polysaccharides by sequential addition of sugars to a membrane-anchored repeating unit, followed by export and polymerization. One of the best studied EPS-producing lactic acid bacterial strains is *L. lactis* NIZO B40 (for a recent review see Kleerebezem *et al.*, 1999), which produces a polymer with the regular repeating unit $\rightarrow$4)[α-Rha*p*-(1$\rightarrow$2)][α-D-Gal*p*-1-PO_4-3]-β-D-Gal*p*-(1$\rightarrow$4)-β-D-Glc*p*-(1$\rightarrow$4)-β-D-Glc*p*-(1$\rightarrow$ (Nakajima *et al.*, 1992b; van Kranenburg *et al.*, 1997). A variety of studies have shown that the backbone of the NIZO B40 repeating unit is assembled from two precursors, UDP-glucose and UDP-galactose, by the activity of specific glycosyltransferases encoded in an *eps* gene cluster

that is encoded on a plasmid (van Kranenburg *et al.*, 1997, 1999b). The subsequent steps in the synthesis of the repeating unit include coupling of the side chain sugars rhamnose and galactosyl phosphate to the galactose of the backbone. Addition of the rhamnose involves a third precursor, dTDP-rhamnose, which is catalyzed by a putative rhamnosyltransferase, and addition of the galactosyl-phosphate is thought to be coupled by a putative glycerophospho-transferase. Both transferases are potentially also encoded in the *eps* cluster (van Kranenburg *et al.*, 1999b).

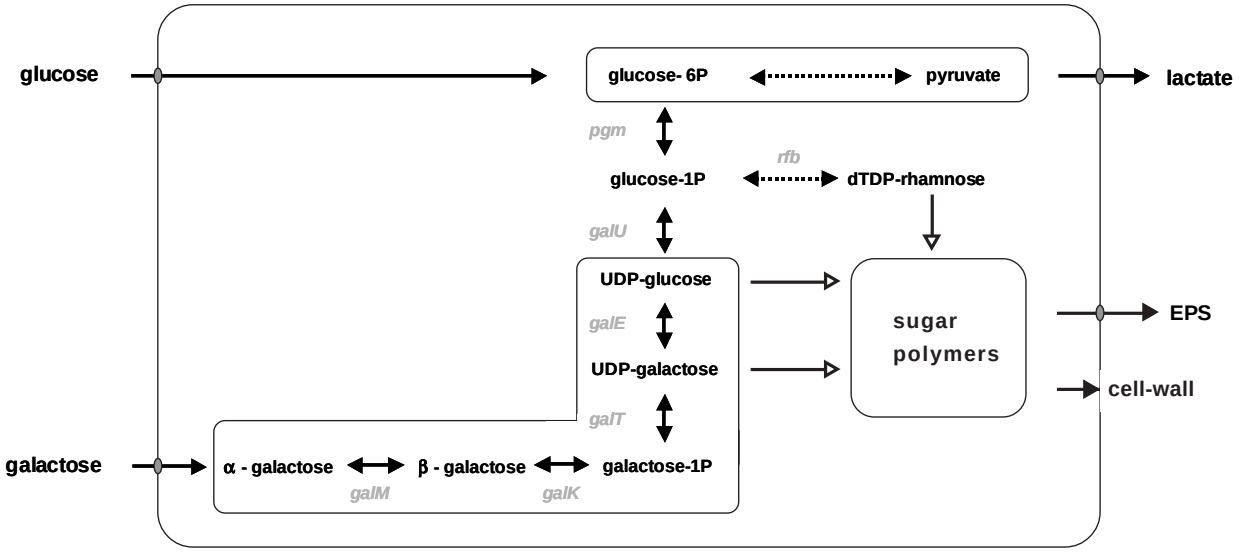

Fig. 4.1. Schematic representation of pathways involved in glucose fermentation via glycolysis (upper box), galactose fermentation via the Leloir pathway (left box), and biosynthesis of EPS in *L. lactis*. Cell membranes are indicated by vertical lines. The following enzymes are involved (encoding genes are indicated): phosphoglucomutase (*pgm*); UDP-glucose pyrophosphorylase (*galU*); UDP-galactose epimerase (*galE*); galactose-1P uridyltransferease (*galT*); galactokinase (*galK*); and galactose mutarotase (*galM*); dTDP-rhamnose biosynthetic enzyme system (*rfb*). Multiple-step reactions are lumped together and are indicated by dotted arrows. The multiple reactions involved in synthesis of sugar polymers are indicated by open arrows.

In addition to specific Eps enzymes encoded in the *eps* gene cluster, EPS biosynthesis requires chromosomally encoded housekeeping enzymes involved in synthesis of the EPS building blocks, the sugar nucleotide precursors. Not only do these sugar nucleotides probably function as precursors for EPS biosynthesis, but they also are involved in biosynthesis of several cell-wall components and are therefore considered essential for growth. The biosynthetic pathways starting from glucose or galactose for formation of the NIZO B40 EPS precursors in *L. lactis*, the sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose, are shown in Fig. 4.1 (Thompson, 1987; van Kranenburg *et al.*, 1999b). Glucose is fermented through the glycolysis step to pyruvate, which in turn is converted to lactate. The glycolytic intermediate glucose-6-phosphate (glucose-6P) is converted to glucose-1P by phosphoglucomutase activity, and this metabolite is subsequently converted to UDP-glucose by UDP-glucose pyrophosphorylase (GalU) activity. Galactose is degraded via the Leloir pathway, leading to the formation of UDP-glucose and UDP-galactose; this involves the gene products encoded by the *galAMKTE* operon (Grossiord *et al.*, 1998). The *galE* gene encodes a UDP-galactose epimerase (GalE), which inter converts these nucleotide sugars.

The potential for using EPS in food is determined by their physical and rheological properties. The factors that influence these properties are structural characteristics, including degree of polymerization, length of side chains, presence of substituents, type of linkages, and sugar composition. Engineering of polysaccharide biosynthesis at the level of the chemical structure of the repeating unit has been performed successfully and has resulted in changes in EPS (Stingele *et al.*, 1999) and also CPS (Chaffin *et al.*, 2000). However, the levels of production of the altered polymers were reduced (Chaffin *et al.*, 2000) or even extremely low (Stingele *et al.*, 1999). Metabolic engineering may be used as a tool to increase EPS production, which requires a detailed understanding of the physiology and genetics of EPS biosynthesis (de Vos, 1996). Although several reports have described the effect of modulation of enzyme activity and its effect on polysaccharide biosynthesis, to our knowledge no study has described the effect on biosynthesis of the sugar nucleotides. For the first time, in this study we evaluated the role of the lactococcal GalU and GalE enzymes in the biosynthesis of NIZO B40 EPS and the EPS precursors UDP-glucose and UDP-galactose by performing overexpression and disruption analyse of the corresponding genes.

Materials and methods

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 4.1. *Escherichia coli* MC1061 (Casadaban and Cohen, 1980), used as a host in cloning experiments, was grown with aeration in TY-broth at 37°C. *L. lactis* was grown without aeration at 30°C in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose (GM17) or in a chemically defined medium (CDM) (Looijesteijn and Hugenholtz, 1999). If appropriate, the media contained chloramphenicol (10 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (2 μg ml^{-1}), or ampicillin (100 μg ml^{-1}). To analyze the effect of gene overexpression, the nisin-controlled expression system was used (De Ruyter *et al.*, 1996a; Kuipers *et al.*, 1998). Briefly, the *L. lactis* cells were grown to an optical density at 600 nm of about 0.5 and then split into two cultures. One nanogram of nisin ml^{-1} was added to one culture, and both cultures were grown for an additional 2 h.

DNA techniques and DNA sequence analysis. Small scale isolation of *E. coli* plasmid DNA was performed as described by Sambrook *et al.* (1989). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Isolation and transformation of *L. lactis* DNA were performed as previously described (de Vos *et al.*, 1989).

Southern blots were hybridized at 65°C with homologous DNA probes, which were labeled by nick-translation using established procedures (Sambrook *et al.*, 1989), and the blots were then washed with a solution containing 0.015 M NaCl and 0.0015 M sodium citrate at 65°C before exposure.

Table 4.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
L. lactis		
MG1363		(Gasson, 1983)
NZ8460	MG1363 *galE::ery*	(Grossiord, 1998)
NZ9000	MG1363 *pepN::nisRK*	(Kuipers *et al.*, 1998)
E.coli		
CGSC4973		*E. coli Genetic Stock Center*[b]
Plasmids		
pCI182	Tet^r	(Hill *et al.*, 1988)
pGEM-T	Ap^r	(Promega)
pUC18	Ap^r	(Yanisch-Perron *et al.*, 1985)
pUC18Ery	Ery^r, integration vector	(van Kranenburg *et al.*, 1997)
pGhost9	Ery^r	(Maguin *et al.*, 1996)
pNZ4000	Eps$^+$	(van Kranenburg *et al.*, 1997)
pNZ4030	Ery^r, Eps$^+$	(van Kranenburg *et al.*, 1997)
pNZ4101	Ap^r, pUC18 derivative containing an MG1363 1.8-kb *Bam*HI-*Pst*I chromosomal DNA fragment carrying the *galU* gene	This work
pNZ4102	Cm^r, pNZ8048 derivative containing a functional lactococcal *galU* gene	This work
pNZ4103	Ery^r, pG$^+$host9 derivative containing a 0.6-kb internal fragment of the *galU* gene	This work
pNZ4130	Tet^r, Eps$^+$, pNZ4030 derivative carrying the *tetM* gene from Tn*919*	This work
pNZ8020	Cm^r, inducible expression vector carrying the *nisA* promoter	(de Ruyter *et al.*, 1996b)
pNZ8048	Cm^r, inducible expression vector carrying the *nisA* promoter	(Kuipers *et al.*, 1998)
pNZ8421	Cm^r, pNZ8020 derivative containing a functional lactococcal *galE* gene	(Grossiord, 1998)
pNZ8460	Ery^r, pUC18 derivative containing a 0.6-kb internal fragment of the *galE* gene	(Grossiord, 1998)

[a]) Eps$^+$, EPS-producing phenotype; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Ery^r, erythromycin resistant; Tet^r, tetracycline resistant.
[b]) Strain CGSC was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

RNA was isolated from *L. lactis* cultures, and Northern analysis was performed as described by Luesink *et al.* (1998). Blots were probed with an internal fragment of the *galU*

gene. To amplify this fragment of the lactococcal *galU* gene, primers 5'-CATTGCCAAA GAAATGTTGCC -3' and 5'-GTCAAGAGGTAACGACCGAT -3' were used in a PCR reaction with *Taq* polymerase and with chromosomal DNA from *L. lactis* MG1363 (Gasson, 1983) as the template.

Automatic double stranded DNA sequence analysis was performed with an ALF*red* DNA sequencer (Pharmacia Biotech, Roosendaal, the Netherlands). Sequence reactions were performed with an Autoread kit, were initiated by using Cy5-labeled universal and reverse primers, and were continued with synthetic primers purchased from Pharmacia Biotech in combination with Cy13-dATP by following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed by using the PC/GENE program, version 6.70 (Intelli-Genetics).

Construction of strains and plasmids. To amplify an internal fragment of the lactococcal *galU* gene, degenerate primers 5'-THCCNGCNGCNGGNYTNGGNACNMGNTTYYT NCCNGCNACNAARGC-3' and 5'-RTCCATNTRRTCRTCNCCNARCATNACNACRAANGG-3' (where H is A, C, or T; N is A, C, G, or T; Y is C or T; M is A or C; and R is A or G) were used in a PCR performed with *Taq* polymerase and with chromosomal DNA from *L. lactis* MG1363 (Gasson, 1983) as the template. The 0.29-kb PCR product generated was sequenced and used as a probe in Southern analysis, it was hybridized with a 1.8-kb *Bam*HI-*Pst*I fragment of the *L. lactis* MG1363 chromosomal DNA, which was cloned in similarly digested pUC18 (Yanisch-Perron *et al.*, 1985), yielding pNZ4101.

To overexpress the *galU* gene, this gene was amplified by PCR performed with *Tth* polymerase, with pNZ4101 as the template DNA, and with primers 5'-ATGCCATGGCAA AACAAACTACTATACCTAACAAAG-3' and 5'-GCGCTCTAGAGCATCAAAAAGAAAA AGCC AATAGGC-3'. The 1-kb PCR product was digested with *Nco*I and *Xba*I (sites underlined in the primer sequences) and inserted under control of the inducible *nisA* promoter into similarly digested pNZ8048; this resulted in pNZ4102, which was transformed into NZ9000 (Kuipers *et al.*, 1998).

To inactivate the *galU* gene by single cross-over recombination, an internal *galU* fragment was obtained by PCR performed with *Taq* polymerase, with pNZ4101 as the template, and with primers 5'-CATTGCCAAAGAAATGTTGCC-3' and 5'- TTTATCACCAACATCATAACG-3'. The 690-bp PCR product was cloned into pGEM-T (Promega Biotech, Roosendaal, The Netherlands). From the resulting plasmid the *galU* internal fragment was isolated as an *Apa*I *Sal*I fragment and cloned in similarly digested pGhost9 (Maguin *et al.*, 1996); this resulted in pNZ4103, which contained a temperature-sensitive replicon which is not functional at 37°C. pNZ4103 was transformed into strains MG1363 and NZ9000, and transformants were subsequently cultured at 37°C. Several erythromycin-resistant (Ery^r) single crossover transformants were selected and were analyzed by Southern analysis. Upon integration, the resulting strain would contain two disrupted copies, one of these copies lacking the 3' end of

galU that encodes the 44 C-terminal amino acids, and the second copy lacking both the 5' translational signals and the first 33 N-terminal amino acids.

galE disruption strain NZ8460 was constructed as described by Grossiord (Grossiord, 1998). Briefly pNZ8460, a pUC18Ery variant containing the *Em* gene of pAMβ and a 0.6-kb internal PCR fragment of the *galE* gene fragment, was transformed into strain MG1363. Ery^r colonies were obtained and were examined by Southern analysis. One of the colonies designated NZ8460, was selected and contained a disrupted copy of the *galE* gene encoding a truncated protein lacking 36 C-terminal amino acids. Since NZ8460 is Ery^r, the EPS-producing capacity could not be introduced into this strain by transformation with pNZ4030 (van Kranenburg *et al.*, 1997) carrying the *eps* operon, which also contains also an *Ery* gene. Therefore, a tetracycline (*tetM*) derivative of pNZ4030 was constructed. To obtain convenient flanking restriction sites, the 4.2-kb *Hin*cII fragment of pCI182 (Hill *et al.*, 1988), containing the *tetM* gene, was subcloned in pUC18 (Yanisch-Perron *et al.*, 1985) digested with *Sma*I. The *tetM* gene was excised from the resulting plasmid, by cutting with *Eco*RI, blunting with the Klenow, and then digesting with *Sph*I. The insert was then ligated to pNZ8020 (de Ruyter *et al.*, 1996b) that had been digested with *Xba*I, end filled with the Klenow, and digested with *Sph*I. Finally, the *tetM* gene was isolated from the resulting plasmid, as an *Xho*I-*Sph*I fragment and was cloned in similarly digested pNZ4000, yielding pNZ4130.

Preparation of cell-free extracts, protein analysis, and enzyme assays. Exponentially grown lactococcal cells (50 ml) were harvested by centrifugation and resuspended in 1 ml of 20 mM sodium phosphate (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The resulting suspension was mechanically disrupted in the presence of zirconium beads (van der Meer *et al.*, 1993). Cell debris was removed by centrifugation. The cell-free extracts (CFE) were each mixed with an equal volume of two-fold concentrated Laemmli buffer, and after boiling 15 μg of each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein content of the CFE was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Enzyme reactions were performed at 30°C in 1-ml mixtures. The UDP-glucose pyrophosphorylase (EC 2.7.7.9) reverse reaction assay was performed as described by Bernstein (Bernstein, 1965). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.8), 14 mM $MgCl_2$, 0.3 mM $NADP^+$, 0.1 mM UDP-glucose, 2.1 U α-phosphoglucomutase (Sigma Aldrich Chemie Gmbh, Steinheim, Germany), 4 U of glucose-6P dehydrogenase (Sigma Aldrich Chemie Gmbh) and CE. The reaction was started by adding 4 mM inorganic pyrophosphate, and the increase in absorbance at 340 nm was determined. UDP-galactose 4-epimerase (EC 5.1.3.2) activity was assayed as described previously (Looijesteijn *et al.*, 1999). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 0.5 mM NAD^+, 0.015 U of UDP-glucose dehydrogenase (Sigma Aldrich Chemie Gmbh) and CE. The reaction was started by adding of 0.2 mM UDP-galactose, and the increase in absorbance at 340 nm was determined.

Intracellular sugar phosphate analysis by ^{31}P-NMR spectroscopy. *L. lactis* NZ8460 harboring pNZ4130 was grown on CDM supplemented with 1% (wt/vol) glucose in a fermentor (New Brunswick Bioflo 2C) at pH 6.5. Ethanol extracts were obtained from a mid-exponential-phase sample (corresponding to 30 mg of protein), and the intracellular sugar phosphates contents of these extracts were determined by ^{31}P nuclear magnetic resonance (^{31}P-NMR) analysis as previously described by Ramos *et al.* (2001). The intracellular metabolite concentrations were calculated by using a value of 2.9 μl per mg of protein for the intracellular volume of *L. lactis* (Poolman *et al.*, 1987).

Sugar nucleotide and EPS exopolysaccharide analysis. Sugar nucleotides were separated from CFE, and sugar nucleotide contents were determined by high-performance liquid chromatography (HPLC) analysis as previously described by Looijesteijn *et al.* (Looijesteijn *et al.*, 1999). EPS were isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (1999). Since the EPS isolation process cannot be strictly controlled, the variation in quantification for individual samples in the same experiment is 5 to 10%, and the variation for individual samples in experiments separated by time is 10 to 20%.

Nucleotide sequence accession numbers. The nucleotide sequences of the *galU* and *galE* genes have been deposited in the GenBank database under accession no. AF304368 and AJ011653, respectively.

Results

Cloning and characterization of the *galU* gene. To identify the *L. lactis* MG1363 *galU* gene, an internal DNA fragment of this gene was obtained by PCR using degenerate primers based on conserved regions in an alignment (PC/GENE package; Intelligenetics, Inc.) of amino acid sequences of the GalU analogues GtaB (accession no. L12272), HasC (U33452), and Cps3U (U15171) from the gram-positive bacteria *Bacillus subtilis*, *Streptococcus pyogenes*, and *S. pneumoniae,* respectively. Sequence analysis of the 0.29-kb PCR product generated revealed a continuous open reading frame (ORF) predicted to encode a protein exhibiting high sequence homology with GalU proteins. A 1.8-kb *Bam*HI-*Pst*I chromosomal DNA fragment of *L. lactis* MG1363 was found to hybridize with this PCR fragment and was subsequently cloned into pUC18, resulting in pNZ4101. Sequence analysis of the insert in pNZ4101 revealed the presence of a 5' truncated ORF and a complete ORF. The truncated ORF putatively encodes a glycerol-3-phosphate dehydrogenase based on homology to the C-terminal part of *B. subtilis* GpdA (45% identity). Translation of the complete ORF resulted in a predicted 313-amino-acid protein with a

calculated molecular mass of 35,002 Da; this protein is referred to here as *L. lactis* GalU, since it exhibited 73% amino acid identity with the GalU homologue *of S. pyogenes* (Crater *et al.*, 1995). The first ATG of the *galU* ORF was preceded by a typical lactococcal Shine Dalgarno sequence (5'-AAGGAG-3') (de Vos and Simons, 1994). A putative promoter region, containing possible –10 (5'-TAATAA-3') and –35 (5'-CTGAA-3') sequences, was found to procede the *galU* coding sequence. An inverted repeat sequence (5'- AAGAAAGAGCCTATTGGCTTT TTCTT-3') and a stretch of six T residues downstream the *galU* coding sequence could function as a rho-independent transcriptional terminator.

To assess the transcriptional organization of the *galU* gene, RNA was isolated from strain MG1363 and used for Northern analysis. An internal fragment of the *galU* gene was generated by PCR, labeled, and used as a DNA probe. This probe hybridized with an approximately 0.9-kb transcript (data not shown). The size of the transcript suggests that the *galU* gene is transcribed as a single monocistronic mRNA from a putative promoter upstream of the *galU* gene and terminates at the putative terminator (see above).

To ascertain that the lactococcal *galU* gene codes for a UDP-glucose pyrophosphorylase, pNZ4101 was introduced into *E. coli galU* mutant CGSC4973, which lacks UDP-glucose pyrophosphorylase activity due to a single base pair substitution in the *galU* gene. While this strain has been shown to be able to grow on glucose, it was not able to grow on galactose (Shapiro, 1966). In contrast, the pNZ4101 transformants could grow on both glucose and galactose, indicating that the *galU* gene of *L. lactis* encodes a functional UDP-glucose pyrophosphorylase.

Modulation of GalU activity. To evaluate the role of reduced GalU activity on UDP-sugar formation and EPS biosynthesis in *L. lactis*, we repeatedly tried to inactivate *galU*. Using a strategy based on a temperature-sensitive replicon, we obtained several integrants, but none of these integrants showed the correct Southern hybridization pattern. These results indicate that integration did not occur in a site-specific manner and left the *galU* gene intact. Moreover, Southern blot analysis of chromosomal DNA obtained from the integrants, using integration vector based probes, revealed that the integration plasmid had been incorporated into the chromosome in a random manner, which eliminated the possibility that a pseudo-*galU* locus or a highly homologous additional copy of the *galU* gene is present in *L. lactis*. Since *galU* appeared to be transcribed as a monocistronic mRNA (see above), polar effects of the intended *galU* disruption were not expected, suggesting that this gene has an essential role in *L. lactis*.

To evaluate the role of increased GalU activity in UDP-sugar formation and EPS biosynthesis in *L. lactis*, we studied the effect of controlled *galU* overexpression by using the nisin-controlled expression system (de Ruyter *et al.*, 1996a; Kuipers *et al.*, 1998). Thus, pNZ8048 derivative pNZ4102 carrying the lactococcal *galU* gene under control of the lactococcal *nisA* promoter was transformed into strain NZ9000. Strain NZ9000 harboring pNZ4102 was grown under inducing and noninducing conditions, and CFE of the cultures were prepared and analyzed

by SDS-PAGE (Fig. 4.2). Growth in the presence of nisin resulted in the appearance of an extra protein band at an apparent molecular mass of approximately 35-kDa, which was the expected size of GalU (see above).

Twenty-fold-higher GalU specific activity was obtained with CFE of the induced cultures than with CFE of the control cultures, demonstrating that controlled and functional overexpression of the *galU* gene occurred (Table 4.2).

Table 4.2. UDP-sugar levels as determined by HPLC and GalU enzyme activities of *L.lactis* NZ9000 (wild-type) and of NZ9000 harboring pNZ4102 (GalU) grown in the presence and in the absence of nisin.

Strain (plasmid)	Nisin (ng ml^{-1})	GalU activity (nmol min^{-1} mg of $protein^{-1}$)	Sugar nucleotides (μmol g of $protein^{-1}$) UDP-glucose	UDP-galactose	UDP-glucose/ UDP-galactose ratio	EPS[a] (mg l^{-1})
NZ9000	0	4.8 ± 0.1	7.6 ± 1	2.8 ± 0.3	2.7	147 ± 7
NZ9000(pNZ4102)	0	4.9 ± 0.1	7.5 ± 0.3	2.6 ± 0.1	2.9	156 ± 9
	1	94 ± 4	60 ± 20	17.5 ± 6	3.4	158 ± 5

[a]) The levels of EPS production were determined after pNZ4030 was transformed.

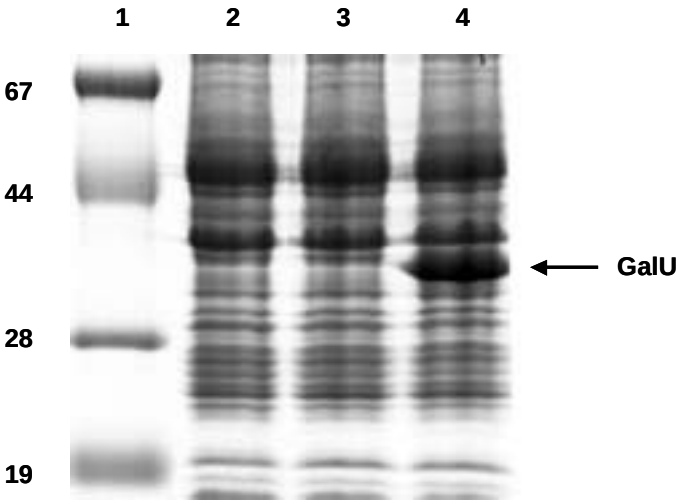

Fig. 4.2. Coomassie blue-stained gel after SDS-PAGE of CFE of *L. lactis* NZ9000 (lane 2) and NZ9000 harboring pNZ4102 grown in the absence (lane 3) or in the presence (lane 4) of nisin. Lane 1 contained a molar weight marker (molecular masses [in kilodaltons] are indicated on the left). The arrow indicates the poition of the overproduced GalU protein.

Effect of *galU* overexpression on UDP-sugar formation and EPS biosynthesis. To study the effect of GalU activity on production of UDP-sugars, which is necessary for production of the NIZO B40 EPS, the concentrations of UDP-glucose and UDP-galactose were determined by performing an HPLC analysis of NZ9000 harboring pNZ4102 grown in the presence and in the absence of nisin. Since the absolute levels of the UDP-sugars were found to be variable, the results of a typical experiment are shown in Table 4.2. Overexpression of *galU* resulted in eightfold increases in the levels of both UDP-glucose and UDP-galactose. Apparently, the endogenous UDP-galactose epimerase (GalE) activity is sufficient to maintain an almost stable ratio of the UDP-sugars in this strain. Although the increased level of GalU activity did not have a significant effect on the level of EPS production (Table 4.2), the results indicate that GalU plays an important role in control of UDP-glucose and UDP-galactose sugar biosynthesis in *L. lactis*.

Effect of *galE* disruption on growth and EPS biosynthesis. The role of the GalE enzyme in growth and EPS biosynthesis was evaluated by analyzing NIZO B40 EPS biosynthesis in *galE* disruption strain NZ8460. As expected, strain NZ8460 was not able to ferment galactose, and CFE of this strain grown on a mixture of glucose and galactose showed no detectable GalE activity (data not shown). The galactose-negative phenotype could be complemented by transforming NZ8460 with pNZ8421 containing the *L. lactis galE* gene under control of its own promoter (data not shown). These results indicate that the *galE* gene was functionally disrupted.

Table 4.3. Maximum growth rates and levels of EPS production for *L.lactis* MG1363 (wild-type) and the *galE* mutant strain NZ8404 ($GalE^-$).

Strain[a]	Carbon source	Growth rate (h^{-1})	EPS[b] (mg l^{-1})
MG1363 (wild-type)	Glc	0.66 ± 0.01[c]	128 ± 6
	Gal	0.19 ± 0.01	91 ± 1
	Glc + Gal	0.66 ± 0.01	107 ± 6
NZ8460 ($GalE^-$)	Glc	0.18 ± 0.01	<1
	Gal	NG[d]	NG
	Glc + Gal	0.52 ± 0.01	44 ± 3

[a]) Both strains harbored pNZ4130 and were grown in CDM supplemented with glucose (Glc), galactose (Gal), or glucose and galactose.
[b]) Levels of EPS production were determined after pNZ4130 was transformed.
[c]) The values are averages based on at least two independent experiments.
[d]) NG, no growth.

EPS producing capacity was introduced into strain NZ8460 by transformation with pNZ4130, which contained the *eps* gene cluster required for biosynthesis of NIZO B40 EPS. Growth of NZ8460 harboring pNZ4130 was analyzed on CDM with different carbon sources (Table 4.3). No difference in growth rate between NZ8460 and its parental strain was observed when cells were grown on a mixture of glucose and galactose. However, NZ8460 was not able to grow on galactose and exhibited a reduced growth rate when it was grown on glucose as the sole carbon source (data not shown). Under the latter culture conditions cell division was also affected, suggesting that GalE has an important role in cell-wall biosynthesis, as first reported by Grossiord (Grossiord, 1998). Remarkably, EPS production was completely absent during growth on glucose (Table 4.3). Moreover, ^{31}P-NMR analysis of extracts obtained from glucose-grown cells of NZ8460 harboring pNZ4130 showed that the intracellular concentration of UDP-galactose was below the level of detection (< 0.2 mM). The concentration of UDP-glucose (0.6 ± 0.12mM) was similar to that obtained with an EPS-producing strain derived from MG1363 (Ramos *et al.*, 2001). Therefore, it is likely that the lack of UDP-galactose leads to an EPS-negative phenotype. However, both EPS production and the growth rate could be restored to intermediate levels by adding galactose to the medium (Table 4.3). The sugar composition of

the EPS produced in a medium containing a mixture of glucose and galactose consisted of glucose, galactose and rhamnose at a ratio of 0.7 (± 0.1) : 1 : 1.9 (± 0.6), which is the expected ratio for NIZO B40 EPS (van Kranenburg *et al.*, 1997), indicating that the *galE* disruption did not alter the specific incorporation of sugar nucleotides.

Taken together, the data show that GalE activity is essential for normal growth and EPS production by cells grown on media in the absence of galactose.

Discussion

EPS contribute to the rheology and texture of fermented products and are therefore of interest for food applications as natural biothickeners. We studied EPS biosynthesis in *L. lactis* NIZO B40, a strain which originated from a very viscous Scandinavian fermented dairy product (van Kranenburg *et al.*, 1997). EPS biosynthesis in *L. lactis* is mediated by proteins encoded by a plasmid-located cluster of *eps* genes that are involved in formation of EPS by sequential addition of sugars to a membrane-anchored repeating unit, export, and polymerization (van Kranenburg *et al.*, 1997, 1999a, 1999b). Besides these specific functions, EPS production also requires household proteins which are involved in biosynthesis of the EPS building blocks, the nucleotide sugars. The model for synthesis of NIZO B40 EPS predicts a requirement for the precursors UDP-glucose, UDP-galactose and dTDP-rhamnose, which are formed from a central intermediate, glucose-1P (Fig. 4.1). To generate tools to target these specific endogenous enzymatic activities, which are potential bottlenecks in sugar nucleotide biosynthesis and subsequent EPS biosynthesis; we cloned and characterized several of the encoding genes. In this study, we evaluated the model for EPS biosynthesis (Fig. 4.1) by analyzing the roles of the *galU* and *galE* gene products in production of UDP-glucose and UDP-galactose from glucose-1P.

Previously, the *galE* gene was cloned from *L. lactis* MG1363 (Grossiord, 1998). Here we describe cloning of the *galU* gene from the same strain. The predicted gene product showed strong homology to UDP-glucose pyrophosphorylases from several bacteria. Evidence for this function was obtained by functional overexpression of the *galU* gene and by functional complementation of an *E. coli galU* mutant. The *galU* gene of *L. lactis* MG1363 is linked to a putative glycerol-3-phosphate dehydrogenase gene. Since these genes are not linked metabolically it is not thought that their genetic linkage has any significance. This hypothesis is supported by the finding that the *galU* gene is expressed as a monocistronic transcript. The *galU* gene in other bacteria has been shown to be genetically linked to genes encoding related metabolic functions. In *S. pyogenes* and *S. pneumoniae* type 3, the *galU* gene is immediately preceded by the *gpsA* gene, which is presumably involved in synthesis of membrane lipids for cell-wall formation. Moreover, in these streptococci another copy of a *galU*-like gene was located in the chromosomal CPS biosynthesis locus (Mollerach *et al.*, 1998). Hence, in *S. pneumoniae*

type 3 residual GalU activity could be measured when the *galU* gene was functionally disrupted, which was probably caused by the second copy of a *galU*-like gene. Interestingly, the *galU* gene of *S. pneumoniae* has been shown to be essential for type 1 or 3 CPS biosynthesis in this organism (Mollerach *et al.*, 1998). Since our attempts to inactivate the *galU* gene in *L. lactis* were unsuccessful, it is likely that the absence of a second *galU* gene copy in *L. lactis* explains the lack of success when we tried to isolate a *galU* mutant.

The role of the Leloir enzyme GalE, which catalyzes interconversion of UDP-galactose and UDP-glucose, in EPS biosynthesis was evaluated by using a *galE* mutant strain. *L. lactis galE* mutant NZ8460 harboring pNZ4130, encoding EPS production, did not produce detectable amounts of EPS when it was cultured on glucose as the sole carbon source. Similar results were obtained for EPS production in *Rhizobium meliloti exoB* (*galE* analogue) (Buendia *et al.*, 1991) and *Erwinia stewartii galE* (Dolph *et al.*, 1988) strains. The EPS-negative phenotype of *L. lactis* could be complemented by adding galactose to the medium; a similar finding was reported for the *E. stewartii galE* strain (Dolph *et al.*, 1988). These results indicate that *galE* plays an essential role in synthesis of UDP-galactose from glucose and thus in EPS biosynthesis. Moreover, the *galE* mutant strain was affected in cell division, which led to formation of long chains of cells when the organism was cultured in medium with glucose as the sole carbon source, as was reported previously by Grossiord (Grossiord, 1998). These results indicate that the *galE* gene is essential not only for EPS production but also for normal cell growth when cells are cultured in media with glucose alone. The effect of *galE* disruption on both EPS production and cell division can probably be explained by a crucial role for UDP-galactose in cell-wall biosynthesis. In glucose-grown wild-type *L. lactis* cells the sugar composition of the poly-saccharide fraction in the cell-wall is 29 mol% glucose, 15 mol% galactose, and 55 mol% rhamnose (Looijesteijn *et al.*, 1999). UDP-glucose is involved in formation of membrane anchors of the lipoteichoic acids (LTA) which are decorated with galactosyl units from UDP-galactose (Delcour *et al.*, 1999). In contrast to UDP-glucose, the precursor UDP-galactose could not be detected in extracts of a glucose-grown culture, suggesting that the lack of UDP-galactose may be a limiting factor for both growth and EPS production. It is possible that the lack of UDP-galactose in the *galE* mutant strain has a significant impact on LTA galactosylation and could therefore lead to inhibition of cell division. Remarkably, a similar mode of growth, long chains of cells, was also observed for *L. lactis* strains that were deficient in autolysin (AcmA) (Buist *et al.*, 1995) or the LTA D-alanylation (DltD) (Duwat *et al.*, 1997). The similarities suggest that the essential role of AcmA in normal cell division (Buist *et al.*, 1995) could depend on alanylation and galactosylation of LTA.

Although growth of the *galE* mutant was completely restored in media containing a mixture of glucose and galactose, the levels of EPS production were not restored to the wild-type level. Since this observation was also made with other independent isolates of the *galE* mutant strain (data not shown), it is not likely that the reduced level of EPS production is caused by mutations in the EPS-plasmid. It has been shown that in *L. lactis* the *gal* genes are subject to CcpA-

mediated catabolite repression, which results in lower levels of transcription of the *gal* operon when cells are grown in the presence of glucose (Luesink *et al.*, 1998). It seems likely that the repressed galactose fermentation in the *galE* mutant strain would lead to reduced availability of UDP-galactose which is preferentially used for growth rather than EPS formation, resulting in an intermediate level of EPS production. This type of control was also found when an EPS-producing *L. lactis* strain was grown in a medium with fructose as the sole carbon source (Luesink *et al.*, 1998).

It has been shown that mutations in the *galE* gene affect the EPS composition of *Rhizobium leguminosarum*, which produces an altered EPS lacking the galactose residue and the substitutions attached to it (Sánchez-Andújar *et al.*, 1997). In addition, *Erwinia amylovora galE* strains were deficient in EPS production but were able to produce LPS, although the LPS had an altered side chain structure (Metzer *et al.*, 1994). These results indicate that in these bacteria it might be possible to change the EPS composition by inactivating the polysaccharide-precursor-forming enzymes. Nevertheless, the sugar composition of the EPS produced by the *galE* strain consisted of glucose, galactose, and rhamnose at the ratio expected for NIZO B40 EPS, indicating that this is not the case for *L. lactis*.

Functional overexpression of the *galU* gene resulted in a twenty-fold increase in enzyme activity and eightfold increases in UDP-glucose and UDP-galactose levels. These results imply that the level of GalU enzyme activity controls the levels of production of UDP-glucose and UDP-galactose in wild-type cells. Apparently, increased GalU activity leading to increased precursor availability did not result in production of more NIZO B40 EPS. This contrasts with a recent report on expression of the *cps3D* and *cps3S* genes from *S. pneumoniae* type 3 in *L. lactis*. This expression resulted in a low level of production of type 3 polysaccharide, which could be increased substantially by coexpression of the *cps3U* gene that encodes a GalU analogue (Gilbert *et al.*, 2000). It has been shown for several bacteria that enzymes involved in sugar nucleotide biosynthesis control EPS production. However, this correlation seems to depend on the type of polysaccharide produced with regard to GalU activity by *L. lactis*. One possible explanation is that the availability of dTDP-rhamnose, which is incorporated as a side chain in NIZO B40 EPS, limits NIZO B40 EPS production. Alternatively, the maximal level of EPS production could also be determined by the activity of the specific EPS biosynthesis machinery encoded by the EPS-plasmid rather than by the level of sugar nucleotides. This hypothesis is supported by the finding that overexpression of the priming glycosyltransferase gene *epsD* resulted in an increase in EPS production (van Kranenburg *et al.*, 1997, 1999c). Elevated expression of all of the biosynthetic *eps* genes would allow evaluation of this alternative for increasing EPS production. Evaluation of the EPS biosynthesis model described here allowed us to assess the role of the GalU and GalE activities in *L. lactis* by using overexpression and disruption studies. We could significantly influence the internal level of UDP-glucose and UDP-galactose, both of which are precursors for EPS biosynthesis as well as for growth. This knowledge is important for targeting bottlenecks in EPS biosynthesis or for creating EPS with novel properties.

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Chapter 5

Chapter

Identification and Functional Characterization of the *Lactococcus lactis rfb* Operon Required for dTDP-rhamnose Biosynthesis

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Abstract

dTDP-rhamnose is an important precursor of cell-wall polysaccharides and rhamnose-containing exopolysaccharides (EPS) in *L. lactis*. We have cloned the *rfbACBD* operon from *Lactococcus lactis* MG1363 comprising four genes involved in dTDP-rhamnose biosynthesis. Northern blot analysis revealed that the operon was transcribed into a monocistronic RNA. When expressed in *E. coli*, the lactococcal *rfbACBD* genes could sustain heterologous production of the *Shigela flexneri* O-antigen, providing evidence for their functionality. Overproduction of the RfbAC or the RfbACBD proteins resulted in doubled dTDP-rhamnose levels in *L. lactis*, indicating that the endogenous RfbAC activities control the intracellular dTDP-rhamnose biosynthesis rate. However, RfbAC overproduction did not result in increased production of a rhamnose-containing lactococcal EPS encoded by pNZ4030. A nisin-controlled-conditional *rfbBD* mutant was constructed which was unable to grow in media lacking the inducer nisin, indicating the essential role of the *rfbACBD* genes in *L. lactis*. Limitation of RfbBD activities resulted in the production of altered EPS in reduced amounts compared to the wild-type strain. Its monomeric sugar composition consisted of glucose, galactose and rhamnose in the molar ratio of 1 : 0.3 : 0.2, and is hereby clearly different from NIZO B40 EPS (1 : 1.7 : 0.8). This implies that the polymerase can recognize and polymerize a repeating unit that differs from that of the wild-type. Biophysical analysis revealed that the altered EPS had a four-fold increased molecular mass while having a two-fold decreased radius of gyration. These results show that the altered EPS are more flexible polymers with changed viscosifying properties. This is the first indication that enzyme activity at the level of central carbohydrate metabolism affects EPS composition.

Introduction

Bacterial polysaccharides can be present in the cell wall as components of the cell envelope. Information about their structure and biosynthesis pathway is fragmented. Glucose, galactose, mannose, N-acetyl-glucosamine, N-acetyl-mannose, and rhamnose are often found as constituents of cell wall polysaccharides (Naumova and Shashkov, 1997).

Rhamnose is a 6-deoxyhexose sugar which is widely distributed in O-antigens of Gram-negative bacteria as part of the lipopolysaccharides (LPS) (Reeves, 1993). Furthermore, it is often found in capsular polysaccharides (CPS), which are covalently bound to the cell wall, and in exopolysaccharides (EPS), which are loosely associated with the cell wall. d-L-TDP-rhamnose is the sugar-nucleotide precursor of these rhamnose moieties and is formed in a four-step reaction from glucose-1-phosphate (glucose-1P). The reaction involves the subsequent enzyme activities of glucose-1P thymidylyl transferase, dTDP-glucose-4, 6-dehydratase, dTDP-4-keto-L-rhamnose-3, 5-epimerase, and dTDP-L-rhamnose synthase encoded by the genes that are commonly designated *rfbABCD*, respectively. These genes have been described in several Gram-negative bacteria such as *Escherichia coli* (Klena and Schnaitman, 1993), *Salmonella enterica* (Li and Reeves, 2000), *Xanthomonas campestris* (Köplin *et al.*, 1993), and *Shigella flexneri* (Macpherson *et al.*, 1994). Various *rfb* mutant strains have been described and these display variable effects on the rhamnose content of the cell wall polysaccharides produced, including a loss of *O*-antigen production (Liu and Reeves, 1994), a reduced amount of LPS production (Klena and Schnaitman, 1993), or LPS production with a reduced amount (Köplin *et al.*, 1993) or a complete lack (Lew *et al.*, 1986) of rhamnose.

In Gram-positive bacteria *rfb* homologues, designated *rml* genes in *Streptococcus mutans* (Tsukioka *et al.*, 1997a; 1997b) and *cps* genes in *Streptococcus pneumoniae* (García *et al.*, 2000; Jiang *et al.*, 2001), were characterized and appeared to play an essential role in the production of sero-type specific, rhamnose-containing CPS antigens. The effect of *rml* mutations was analyzed in *S. mutans* and resulted in a changed cell wall composition, lacking rhamnose. Moreover, the mutations led to a complete loss of production of the sero-type specific O-antigen (Tsukioka *et al.*, 1997a, 1999b). *S. pneumoniae cps19fL and N* mutants exhibited a so-called rough phenotype and lost the capacity to produce CPS, indicating an essential role for the *rfb* analogues in CPS-19F production (Morona *et al.*, 1997).

Various lactic acid bacteria (LAB), including lactobacilli (Hall and Knox, 1965; Coyete and Ghuysen, 1970; Douglas and Wolin, 1971; Wicken *et al.*, 1983), streptococci (Schleifer and Kilpper-Bältz, 1987), and lactococci (Looijesteijn *et al.*, 1999; Sijtsma *et al.*, 1991), characteristically contain rhamnose in their cell walls. Rhamnose is a major component of the lactococcal cell-wall (Looijesteijn *et al.*, 1999; Sijtsma *et al.*, 1991) and has been suggested to be the primary binding site for certain bacteriophages (for recent review see Forde and Fitzgerald, 1999). Moreover, it is also found as a component of the EPS produced by *L. lactis*

SBT0495 (Nakajima *et al.*, 1992), NIZO B40 (van Kranenburg *et al.*, 1997; van Casteren *et al.*, 1998), and NIZO B39 (van Casteren *et al.*, 2000). Since EPS-producing LAB are applied in the food industry where the *in situ* produced EPS determines dairy-product properties like texture, these EPS could provide a potential new source for food-grade biothickeners (Duboc and Mollet, 2001).

Here we describe the cloning and functional analysis of the *rfb* operon involved in dTDP-L-rhamnose biosynthesis in *L. lactis*, including complementation of an *E. coli rfb* mutant, and the effects of homologous overexpression of the *rfb* genes in *L. lactis* on dTDP-rhamnose synthesis. In contrast to Gram-negative bacteria, *rfb* genes are essential in *L. lactis* as is illustrated by using a *rfbBD* conditional mutant. In addition, the *rfbBD* conditional mutant produced altered EPS with a modified sugar composition and novel physical characteristics.

Materials and methods

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 5.1. *Escherichia coli* MC1061 (Casadaban and Cohen, 1980), which was used as a host in cloning experiments, was grown with aeration in TY-broth at 37°C. *L. lactis* was grown without aeration at 30°C in M17 broth (Merck, Darmstadt, Germany), supplemented with 0.5% (wt/vol) glucose or in a chemically defined medium (CDM) (Looijesteijn and Hugenholtz, 1999). If appropriate, the media contained chloramphenicol (10 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (2.5 μg ml^{-1}), kanamycin (25 μg ml^{-1}) or ampicillin (100 μg ml^{-1}). To analyze the effect of gene overexpression the nisin-controlled expression system (NICE) was used (de Ruyter *et al.*, 1996, Kuipers *et al.*, 1998). Briefly, *L. lactis* cells were grown till an optical density at 600 nm of about 0.5 and then split into two cultures. One ng ml^{-1} of nisin was added to one of the two cultures, and both cultures were grown for an additional 2 h.

DNA manipulations and DNA sequence analysis. Small scale isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Isolation and transformation of *L. lactis* DNA were performed as previously described (de Vos *et al.*, 1989).

Automatic double-stranded DNA sequence analysis was performed on both strains with an ALF*red* DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands). Sequence reactions were performed with an Autoread kit, were initiated by using Cy5-labeled universal and reverse primers, and were continued with synthetic primers purchased from Pharmacia

Biotech in combination with fluorescein-15-dATP by following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed by using the PC/GENE program, version 6.70 (Intelli-Genetics).

Construction of strains and plasmids. To amplify an internal fragment of the lactococcal *rfbA* gene, degenerate primers 5'-TAYGAYAARCCNATGATHTAYTAYCC -3' and 5'-RTGNGTNCC NGTRTCNARCCA -3' (where H is A, C, or T; N is A, C, G, or T; Y is C or T; and R is A or G), based on conserved regions in an alignment (PC/GENE package; Intelli-Genetics, Inc.) of amino acid sequences of RfbA analogues RmlA and CPS19FL from *S. mutans* (accession no. D78182) and *S. pneumoniae* (U09239.1), were used in a PCR reaction with chromosomal DNA from *L. lactis* MG1363 (Gasson, 1983) as a template. The 0.6-kb PCR product generated was cloned in pGEM-T (Promega) and sequence analysis revealed a continuous open reading frame (ORF), predicted to encode a protein exhibiting high sequence homology with RfbA proteins. The resulting plasmid was used as a probe in Southern analysis. This fragment hybridized with a 3.8 kb-*Sac*I/*Eco*RI fragment and a 2.4-kb *Hin*dIII fragment of the *L. lactis* MG1363 chromosomal DNA, which were cloned in similarly digested pUC18 (Yanisch-Perron *et al.*, 1985), yielding pNZ4104 and pNZ4106 respectively. Sequence analysis of the inserts revealed the presence of four open reading frames. These ORF's were predicted to encode proteins of 289, 197, 350, and 300 amino acids that exhibited high sequence identity to RfbA, C, B, and D proteins, respectively.

The *rfbAC* overexpression plasmid was constructed by cloning a 2.0-kb *Pst*I-*Hin*dIII fragment of pNZ4106, containing *rfbAC* into similarly digested pNZ8048 (Kuipers *et al.*, 1998), yielding pNZ4115. The *rfbAC* fragment cloned in pNZ4115 contains a putative termination sequence upstream of the *rfbA* coding sequence that hampered expression of the *rfbAC* genes (data not shown). Therefore, this sequence was deleted using a PCR strategy. The 5' coding region of rfbA was amplified using pNZ4115 as template DNA combined with the primers 5'-AACTGCAGGCTAATTAATTATGATTATGGAGGTCC -3' and 5'-AGCATATTGAAGATT GATACC -3'. The 223-bp PCR product generated, was cloned into pNZ4115 using the *Pst*I restriction site introduced in the forward primer (underlined) and the *Sac*I restriction site present in the PCR product generated, yielding the functional *rfbAC* overexpression construct pNZ4116 (see results). The *rfbACBD* overexpression plasmid was constructed by cloning the 3.8-kb *Sac*I-*Eco*RI fragment of pNZ4104, containing the *'rfbACBD* ORF's into *Pst*I-*Hin*dIII digested pNZ4116, after blunting the *Eco*RI and *Hin*dIII sticky ends using Klenow, yielding pNZ4117. The *rfbB* gene was amplified by PCR using pNZ4104 as template DNA combined with the primers 5'-CATGCCATGGCAACTGAATTTAAAAATATCGTTGTGACAG -3' and 5'-GCGCTCTAGAG CTAGGATTTCATCAGCAAATTTTGG -3'. The 1.1-kb PCR product generated was cloned into pNZ8048 (Kuipers *et al.*, 1998) by making use of the *Nco*I and *Xba*I restriction sites that were introduced by the primers used (underlined), yielding the *rfbB* overexpression plasmid pNZ4118. The plasmid pNZ4116, pNZ4117 or pNZ4118 was transformed into *L. lactis* strain NZ9000 (Kuipers *et al.*, 1998). The EPS producing capacity was introduced in NZ9000 harboring

Table 5.1. Bacterial strains and plasmids, their relevant characteristics and sources used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
L. lactis		
MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	(Gasson, 1983)
NZ9000	MG1363 *pepN::nisRK*	(Kuipers *et al.*, 1998)
E. coli		
MC1061		(Casabadan and Cohen, 1980)
Sϕ874	*E. coli* K12 analogue	(Neuhard and Thomassen, 1976)
Plasmids		
pGEM-T	Ap^r	(Promega)
pGhost8	Ery^r	(Maguin *et al.*, 1996)
pK194	Km^r	(Jobling and Holmes, 1990)
pUC18	Ap^r	(Yanisch-Perron *et al.*, 1985)
pUC19	Ap^r	(Yanisch-Perron *et al.*, 1985)
pUC18Ery	Ap^r, Ery^r	(van Kranenburg *et al.*, 1997)
pPM2213	Ap^r , contains the *S. flexneri rfb* region and directs *S. flexneri* 4 O-antigen production in *E. coli*	(Macpherson *et al.*, 1991)
pPM2716	Ap^r, pPM2213 derivative lacking the *S. flexneri rfbBDAC* genes	(Macpherson *et al.*, 1994)
pNZ4000	43-kb plasmid encoding EPS production	(van Kranenburg *et al.*, 1997)
pNZ4030	Ery^r, EPS^+, 27-kb derivative of pNZ4000	(van Kranenburg *et al.*, 1997)
pNZ4104	Ap^r, pUC18 derivative containing the MG1363 3.8-kb *Sac*I/*Eco*RI chromosomal DNA fragment with the *'rfbACBD* genes	This work
pNZ4105	Ap^r, Ery^r, pUC18Ery derivative containing the 1.2-kb *Sph*I/*Spe*I fragment with the '*rfbAC'* genes	This work
pNZ4106	Ap^r, pUC18 derivative containing the MG1363 2.4-kb *Hin*dIII chromosomal DNA fragment with the *rfbAC* genes	This work
pNZ4107	Ap^r, Ery^r, pUC18Ery derivative containing the *Acc*I/*Eco*RI fragment with the *rfbD* gene.	This work
pNZ4108	Ap^r, Ery^r, pUC18Ery derivative containing the *Acc*I/*Eco*RI fragment with the *rfbD* gene and the *Hin*dIII fragment with the *'rfbAC* genes of pNZ4104.	This work
pNZ4109	Ap^r, Ery^r, Tet^r derivative of pNZ4108 containing the *tetR* gene from pGost9 pNZ4110	This work
pNZ4110	Km^r, derivative of pK194 carrying the lactococcal *rfbACBD* genes, transcriptionally fused to the *nisA* promoter	This work
pNZ4111	Ap^r, derivative of pUC19 with the 1.4-kb*Stu*I/*Sac*I fragment from pNZ4118 containing the *Cm* terminator sequence and the *nisA* promotor	This work
pNZ4112	Ap^r, pNZ4111 derivative containing the *Eco*RI/*Hin*dIII fragment of pNZ4104 with the *rfbAC* genes	This work
pNZ4113	Ap^r, Ery^r, pNZ4112 derivative containing the *ery* gene from pUC18Ery	This work
pNZ4114	Ap^r, Ery^r, Tet^r, derivative of pNZ4113 containing the *tetR* gene from pGhost8	This work
pNZ4115	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbAC* genes under control of the putative *rfb* promoter	This work
pNZ4116	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbAC* genes, transcriptionally fused to the *nisA* promoter	This work
pNZ4117	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbACBD* genes, transcriptionally fused to the *nisA* promoter	This work
pNZ4118	Cm^r, derivative of pNZ8048 carrying the lactococcal *rfbB* gene, translationally fused to the *nisA* promoter	This work
pNZ8048	Cm^r, lactococcal cloning and expression vector with the *nisA* promoter upstream of a multiple cloning site	(Kuipers *et al.*, 1998)

[a]) EPS^+, EPS-producing phenotype; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant, Ery^r, erythromycin resistant, Tet^r, tetracycline resistant; Km^r, kanamycin resistant.

pNZ4116, pNZ4118, or pNZ4117 by electroporation of plasmid pNZ4030 (van Kranenburg *et al.*, 1997), which contains the NIZO B40 *eps* gene cluster.

To ascertain that the *L. lactis rfbACBD* operon encodes a functional dTDP-rhamnose biosynthesis pathway a 3.9-kb *Sca*I-*Pst*I fragment of pNZ4117 (see below) was cloned into pK194 (Jobling and Holmes, 1990). The resulting plasmid pNZ4110 was transformed to *E. coli* Sϕ874 containing pPM2716 (Macpherson *et al.*, 1994).

Several strategies were employed to knock-out one of the *rfb* genes. To knock out the *rfbA* gene by a single cross-over plasmid integration a 1.2-kb PCR fragment containing the 3'-end of RfbA and the 5' end of RfbC was amplified using the primers 5'-TATCTATGAT AAACCAATGATTTATTATC-3' and 5' GCCCAGTAATCATTAACCAG-3' and cloned into pGEMT (Promega). From the resulting plasmid a 1.2-kb *Sph*I-*Spe*I fragment was cloned into pUC18Ery (van Kranenburg *et al.*, 1997). The resulting plasmid pNZ4105 was transformed to *L. lactis* NZ9000, but no erythromycin resistant (Ery^r) colonies could be obtained.

To knock-out the *rfbB* gene by a double crossing over gene replacement strategy an integration plasmid was constructed, which contained an erythromycin-resistance gene cassette flanked by the up- and downstream regions of the *rfbB* gene. To this end, a 1.1-kb *Acc*I-*Eco*RI fragment of pNZ4104 containing the *rfbB* downstream region was cloned into similarly digested pUC18Ery (van Kranenburg *et al.*, 1997), yielding pNZ4107. Subsequently, a 1.6-kb *Hin*dIII fragment of pNZ4104, containing the *rfbB* upstream region, was cloned into similarly digested pNZ4107, yielding pNZ4108. To facilitate direct double cross-over transformant selection an additional selection marker *tetR*, which was isolated as a *Sma*I-*Ecl*136 fragment from pGhost8 (Maguin *et al.*, 1996), was cloned into the *Sma*I restriction site of pNZ4108. The resulting plasmid pNZ4109 was transformed to *L. lactis* NZ9000 and *L. lactis* NZ9000 harboring pNZ4118. Ery^r colonies were screened by replica plating on GM17 plates containing tetracycline or erythromycin and one ng ml^{-1} nisin. Ery^r Tet^s integrants could only be obtained in *L. lactis* NZ9000 harboring pNZ4118. Southern analysis confirmed that all of these Ery^rTet^s integrants contained a disrupted copy of the *rfbB* gene on the chromosome and one colony, designated NZ4109 was selected for further analysis.

To be able to make a *rfbBD* conditional mutant a 1.4-kb *Stu*I-*Sac*I fragment of pNZ4118, including a chloramphenicol (*Cm*) gene derived termination sequence, the *nisA* promoter and the RfbB ORF was cloned in pUC19 (Yanisch-Perron *et al.*, 1985) digested with *Sma*I and *Sac*I, yielding pNZ4111. In pNZ4111 the 1.5-kb *Hin*dIII-*Eco*RI fragment of pNZ4104, containing *'rfbAC*, was cloned, yielding pNZ4112. The erythromycin-resistance gene cassette was isolated as a 1.2-kb *Hin*dIII-*Kpn*I fragment (former site filled in with Klenow) from pUC18Ery (van Kranenburg *et al.*, 1997) and cloned in *Sma*I-*Kpn*I digested pNZ4112, yielding pNZ4113. Finally, to facilitate direct double cross-over transformant selection an additional selection marker *tetR*, which was isolated as a *Sma*I-*Ecl*136 fragment from pGhost8 and was cloned in the *Hin*dIII (after filling of the cohesive ends using Klenow) restriction site of pNZ4113. The resulting plasmid pNZ4114 was transformed to *L. lactis* NZ9000. Colonies were replica plated on plates with GM17 and

tetracycline or erythromycin. Tet^{r}Ery^s colonies obtained were further analyzed by Southern analysis, and one single colony, designated NZ4114, was selected that contained the *rfbAC* coding sequence, followed by the desired integration of the tetracycline resistance gene cassette, the *Cm* gene derived termination sequence and the *nisA* promoter followed by the *rfbB* coding sequence.

Preparation of cell-free extracts and protein analysis. Lactococcal cells (50 ml) were harvested by centrifugation (3,500 x *g*, 10 min, 4°C) and cell pellets were resuspended in 1 ml of 20 mM sodium-phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. These suspensions were mechanically disrupted by bead-beating in the presence of Zirconium beads (van der Meer *et al.*, 1993) and cell debris was removed by centrifugation (3,500 x *g*, 10 min, 4°C). The protein content of the cell-free extract (CFE) was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

The lactococcal CFEs were each mixed with an equal amount of two-fold-concentrated Laemmli buffer, and after boiling, 15 μg of each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Northern, Southern and Western blot analysis. Southern blots were hybridized at 65°C with homologous DNA probes, which were labeled by nick-translation using established procedures (Sambrook *et al.*, 1989), and the blots were subsequently washed with a solution containing 0.015 M NaCl and 0.0015 M sodium citrate at 65°C before exposure.

RNA was isolated from *L. lactis* cultures, and Northern analysis was performed as described by Luesink *et al.* (1998). Blots were probed with internal fragments of the *rfbA* and *rfbD* genes. The internal fragment of the lactococcal *rfbA* gene was isolated as a 0.4-kb *Eco*RV-*Afl*II fragment from pNZ4105 and the internal fragment of the lactococcal *rfbD* gene was isolated as a 0.7-kb *Eco*RI-*Hin*dIII fragment from pNZ4104.

For Western blot analysis of *E. coli* protein samples were prepared by harvesting of one ml of cell culture followed by resuspension in 100 μl distilled water. Proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) (Towbin *et al.*, 1979) using electroblot equipment according to the instructions of the manufacturer (LKB 2051 Midget Multiblot). Filters were probed with rabbit antiserum raised against *S. flexneri O*-antigen (Sifin, Berlin, Germany), used at a dilution 1 : 2500. Primary, *O*-antigen bound antibodies were detected by using goat anti-rabbit, peroxidase-conjugated antibodies and a peroxidase specific reaction according to the instructions of the manufacturer (Pierce, Illinois, USA).

Enzyme assays. Enzyme reactions were performed at 30°C in a total volume of 1-ml mixtures with freshly prepared CFE in various concentrations. The formation of NAD(P)(H) was determined by measuring the change of the absorbence at 340 nm. Given values are the means of at least

two independent measurements. The blank contained the reaction buffer, cofactors and the substrate, but lacked the CFE.

The glucose-1-phosphate thymidylyl transferase (RfbA; EC 2.7.7.24) reverse reaction assay was based on the assay described by Bernstein (1965). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.8), 8 mM $MgCl_2$, 0.3 mM $NADP^+$, 2.1 U of α-phopsphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, 4 mM inorganic phosphate and CFE. The reaction was started by the addition of 0.1 mM dTDP-glucose.

The overall activity of dTDP-glucose 4,6 dehydratase (RfbB; E.C. 4.2.1.46), dTDP-4-keto-6-deoxy-D-glucose-3,5-epimerase (RfbC; E.C.5.1.3.13) and dTDP-4-keto-L-rhamnose reductase (RfbD; E.C. 1.1.1.133) enzymes was assayed in a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 0.5 mM NADH, and CFE. 0.3 mM dTDP-glucose was added to start the reaction (adapted from Grobben *et al.*, 1996).

The dTDP-D-glucose 4,6-dehydratase (RfbB) (E.C. 4.2.1.46) reaction (in a volume of 700 µl) contained 50 mM Tris-HCl buffer (pH 8.0) and CFE. The reaction was started with the addition of 43 mM dTDP-glucose. In a time-range 75 µl samples of the reaction mixture were taken and added to 600 µl of 0.5 M NaOH. After 10 min. of incubation the formation of dTDP-4-keto-6-deoxy-D-glucose was determined at 320 nm. The molar absorption coefficient of dTDP-4-keto-6-deoxy-D-glucose that was used to calculate specific activity levels is $6.5 * 10^3$ l $mol^{-1}cm^{-1}$ (Zarkowsky and Glaser, 1969).

Sugar nucleotide and EPS analysis. Sugar nucleotides were separated from CFE, and individual sugar nucleotide contents were determined by high-performance liquid chromatography as previously described by Looijesteijn *et al.* (1999). The values reported are the average of at least two independent determinations. EPS were isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (1999). Molecular mass and radius of gyration were determined via the proGram Insight II (Biosym MS I, USA). The intrinsic viscosity was calculated as described by Tuinier *et al.* (1999) using the equation:

$$[n] = [10\pi (R_g/1.27)^3 N_{AV}] / 3 * M$$

in which N_{AV} is Avogadro's number, R_g is the radius of gyration and M is the molar mass.

Isolation of cell-wall sugars and characterisations of EPS and cell-wall sugars. The isolation of cell-wall sugars was performed as described by Looijesteijn *et al.* (1999). Lactococcal cells (50 ml) grown in CDM were harvested in stationary phase by centrifugation (3,500 x *g*, 10 min, 4°C) and washed twice with 0.85 % (wt/vol) NaCl at 4°C. After disruption by French press (2 x 18,000 psi) whole cells were removed by centrifugation (3,500 x *g*, 10 min, 4°C) and the supernatant was centrifuged (200,000 x *g*, 60 min, 4°C) to harvest cell-envelopes. The crude cell-envelope fraction obtained was resuspended in 50 mM MOPS buffer pH 7 containing 140

µg of RNase and 100 µg of DNase per ml and incubated for 90 min at 37°C. Cell envelopes were re-isolated by centrifugation (200,000 x *g*, 60 min, 4°C) and resuspended in 0.5 mM MOPS buffer pH 7 with 2% SDS and incubated at 70°C for 1 h. After centrifugation (200,000 x *g*, 60 min, 4°C), the pellet was washed twice with distilled water to remove SDS and subsequently freeze-dried, which resulted in a purified cell-wall fraction. Isolated EPS or cell-walls were hydrolyzed in 4 M HCl for 30 min. at 100°C. Samples were dried under vacuum and dissolved in distilled water. The monomeric sugar composition after hydrolysis was determined by HPLC (Van Riel and Olieman, 1991). The values presented are averages based on at least two independent experiments.

Nucleotide sequence accession numbers. The nucleotide sequences of the *rfbACBD* genes have been deposited in the GenBank under accession of no AF458777.

Results

Cloning and functional analysis of the *rfbACBD* gene cluster. The lactococcal *rfb* genes are organized in an operon-like structure and are all preceded by typical Shine-Dalgarno sequences. A putative promoter region, containing possible –10 (5'-TATAAT-3') and –35 (5'-TTGTGT-3') sequences was found to precede the *rfbA* coding sequence. An inverted repeat sequence (5'-TAATGACTTTGTCATTA-3') followed by an A/T rich region downstream the *rfbD* coding sequence could function as a rho-independent transcriptional terminator.

To assess the transcriptional organization of the *rfb* gene cluster, RNA was isolated from strain *L. lactis* MG1363 and used for Northern analysis. Internal fragments of the *rfbA* and *rfbD* genes were generated by PCR, labeled and used as DNA probes. Both probes hybridized with a transcript of approximately 3.8-kb. No other transcripts were detected. The size of the *rfb*-specific transcript showed that the *rfb* genes are transcribed as a single 3.8-kb polycistronic mRNA starting from the postulated promoter upstream the *rfbA* gene and terminating at the putative terminator (see above).

To ascertain whether the *L. lactis rfbACBD* operon encodes a functional dTDP-rhamnose biosynthesis pathway these genes were cloned into pK194 (Jobling and Holmes, 1990). The resulting plasmid, pNZ4110, was transformed to *E. coli* Sϕ874 containing pPM2716, which is a derivative of pPM2213. pPM2213 contains the complete *S. flexneri* 4 *rfb* region and directs the expression of *S. flexneri* 4 *O*-antigen production in *E. coli*. The difference that is introduced in pPM2716 relative to pPM2213 is that the *S. flexneri rfbBDAC* genes have been deleted (Macpherson *et al.*, 1994). Cells of *E. coli* Sϕ874 harboring pPM2213, pPM2716, pPM2716 and pNZ4110, or pPM2716 and pK194 were subjected to Western blot analysis using rabbit

antiserum raised against *S. flexneri O*-antigen (Fig. 5.1). Immunoreactive *O*-antigen could only be detected in cells harboring pPM2213 or pPM2716 and pNZ4110, indicating that the *L. lactis rfbACBD* operon is functional. Therefore, we conclude that RfbA is a glucose-1P thymidylyl transferase, RfbB is a dTDP-glucose-4,6-dehydratase, RfbC is a dTDP-4-keto-L-rhamnose-3,5-epimerase, and RfbD is a dTDP-L-rhamnose synthase.

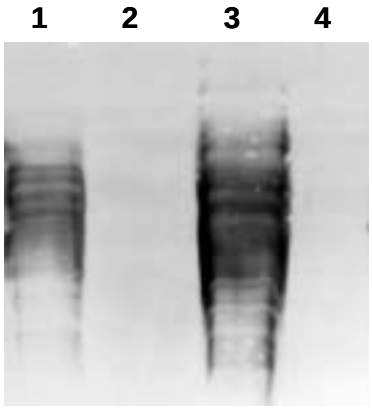

Fig. 5.1. Complementation of *S. flexneri rfbBDAC* by *L. lactis rfbACBD*. *E. coli* lysates were separated by SDS-PAGE and subjected to Western blot analysis using rabbit antiserum raised against *S. flexneri* O-antigen. Lanes: 1, *E. coli* Sϕ874 containing pPM2213; 2, *E. coli* Sϕ874 containing pPM2716; 3, *E. coli* Sϕ874 containing pPM2716 and pNZ4110; 4, *E. coli* Sϕ874 containing pPM2716 and pK194.

Physiological effects of rfb overexpression. To evaluate the control of Rfb activity in sugar nucleotide formation and EPS biosynthesis in *L. lactis*, pNZ8048 derivatives carrying the lactococcal *rfb* genes under control of the lactococcal *nisA* promoter were transformed to NZ9000, which allows the use of the nisin-controlled expression (NICE) system (De Ruyter *et al.*, 1996, Kuipers *et al.*, 1998). Strain NZ9000 harboring the pNZ8048 derivatives carrying *rfbACBD* (pNZ4117), *rfbAC* (pNZ4116) or *rfbB* (pNZ4118) under control of the lactococcal *nisA* promoter (see Table 5.1) were grown in the presence or absence of nisin and CFE's of these cultures were analyzed by SDS-PAGE (Fig. 5.2). Nisin-mediated induction of NZ9000 harboring pNZ4116 (*rfbAC*) resulted in the appearance of additional protein bands with molecular masses of approximately 32- and 22-kDa, which are the expected sizes of RfbA and RfbC, respectively (lane 2). In analogy, nisin-mediated induction of strain NZ9000 harboring pNZ4118 (*rfbB*) resulted in the appearance of an additional protein band with a molecular mass of approximately 40-kDa, which is the expected size of RfbB (lane 4). Furthermore, nisin-mediated induction of NZ9000 harboring pNZ4117 (*rfbACBD*) resulted in the appearance of additional protein bands with molecular masses of approximately 40-, 32-, and 22-kDa (lane 7). The RfbA and RfbD proteins appeared to co-migrate at an apparent molecular mass of 32-kDa, which can be deduced from the intensity increase of this band relative to the RfbC (22-kDa) band when *rfbACBD* is expressed instead of *rfbAC*. This observation strongly suggests that in addition to the RfbA protein also the RfbD protein, which has approximately the same predicted molecular mass as RfbA, is overproduced in the RfbACBD overproduction strain.

The RfbA enzyme activity level was determined in the CFE of the wild-type strain (21 ± 0.5 nmol mg of protein^{-1}) and in the induced cultures of RfbAC (1340 ± 200 nmol mg of protein^{-1}) and RfbACBD (268 ± 28 nmol mg of protein^{-1}) overproducing strains, indicating that 67- and 13-fold

overexpression levels of RfbA were achieved, respectively. The lower RfbA activity measured in the RfbACBD-overproduction strain compared to the RfbAC-overproduction strain is probably due to the reverse RfbA enzyme activity assay in which RfbA and RfbB are competing for the same substrate, dTDP-glucose. The RfbB enzyme activity level was 60- and 45-fold increased, respectively, relative to the wild-type level (28 ± 1 nmol mg of protein^{-1}) in the induced RfbB (1570 ± 150 nmol mg of protein^{-1}) and RfbACBD (1160 ± 100 nmol mg of protein^{-1}) overproducing strains. Moreover, the RfbBCD enzyme activity level was increased 28-fold for the RfbACBD (508 ± 70 nmol mg of protein^{-1}) overproducing strain compared with the wild-type level (18 ± 5 nmol mg of protein^{-1}). Remarkably, in contrast to other bacteria the lactococcal RfbD activity could only be measured using NADH instead of NADPH in the RfbBCD enzymatic assay (data not shown), indicating that the lactococcal RfbD activity requires NADH as a cofactor. These increased enzyme activities demonstrate that controlled and functional overexpression of the *rfbACBD* genes was achieved.

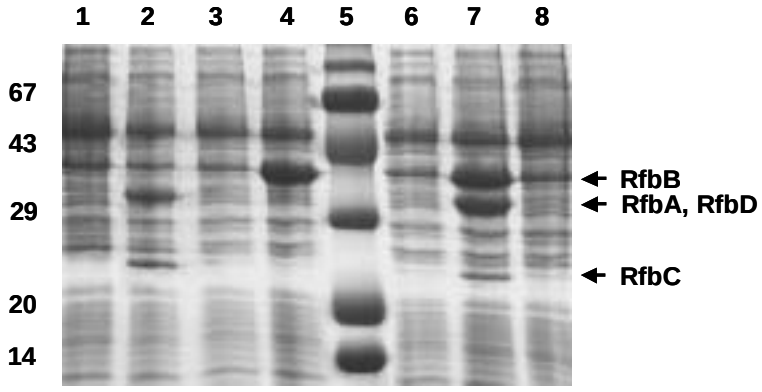

Fig. 5.2. Coomassie blue-stained gel after SDS-PAGE of CFE of *L. lactis* NZ9000 and pNZ8048 (lane 8), pNZ4116 (RfbAC, lanes 1 and 2), pNZ4118 (RfbB, lanes 3 and 4), or pNZ4117 (RfbACBD, lanes 6 and 7) grown in the presence (lanes 2, 4, and 7) or absence (lanes 1, 3, and 6) of nisin. The arrows indicate the positions of the overproduced proteins.

The control of Rfb activity on sugar nucleotide formation and glucose-1P pool dissipation was evaluated in the *rfb* overexpression strains. Functional overexpression of the *rfb* genes resulted in a maximal growth rate that was not significantly different from that of the wild-type growth (data not shown). Furthermore, the overexpression of the *rfb* genes did not influence the absolute level of UDP-glucose or UDP-galactose (data not shown). In contrast, the overexpression of *rfbAC* and *rfbACBD* resulted in a doubling of the intracellular dTDP-rhamnose levels (Fig. 5.3) while the dTDP-glucose level in these strains remained the same (data not shown). These results demonstrate that RfbAC activities have a high control on dTDP-rhamnose biosynthesis in wild-type cells.

To evaluate the effect of *rfb* overexpression on EPS production, the EPS producing capacity was introduced into the *rfb* overproducing strains by transformation of these strains with pNZ4030, which contains the *eps* gene cluster of NIZO B40 EPS. Increased Rfb activity levels had no effect on the NIZO B40 EPS production level (data not shown). Apparently, although increased Rfb activity levels resulted in increased dTDP-rhamnose levels these modulations did not affect growth rate or NIZO B40 EPS production.

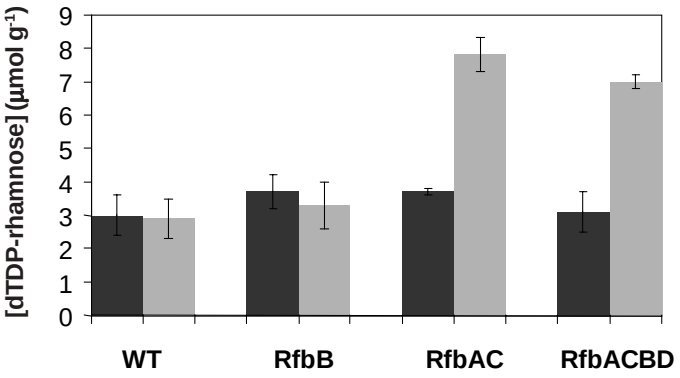

Fig. 5.3. dTDP-rhamnose levels of *L. lactis* NZ9000 harboring pNZ8048 (WT), pNZ4118 (RfbB), pNZ4116 (RfbAC) or pNZ4117 (RfbACBD) grown in the absence (dark grey bar) and in the presence (grey bar) of 1 ng ml^{-1} nisin.

Effect of *rfb* mutation on growth and EPS production. To evaluate the effect of reduced Rfb activity on growth and EPS biosynthesis in *L. lactis*, we tried to inactivate the *rfb* genes. Several attempts to disrupt the *rfbA* gene by single cross-over plasmid integration using pNZ4105 failed. Moreover, attempts to select mutant strains in which the *rfbB* gene was replaced by an erythromycin-resistance gene cassette by direct double cross over, using the non-replicative plasmid pNZ4109, were unsuccessful. All erythromycin-resistant colonies selected using the latter strategy appeared to be single cross-over integrants. Southern analysis of these strains revealed that in all cases the single cross-over plasmid integration had taken place downstream of the *rfbB* gene, thereby leaving the *rfb* operon intact. These results strongly suggest that the *rfb* genes fulfill an essential role in *L. lactis*. This suggestion was corroborated by the finding that the desired *rfbB:ery* strain could be obtained by transformation of pNZ4109 to *L. lactis* cells harboring an additional copy of the *rfbB* gene *in trans* on a replicating plasmid, pNZ4118, that harbors *rfbB* under control of the *nisA* promoter. However, the strain obtained following this rescue strategy was still able to grow in the absence of nisin, suggesting that the level of RfbB protein could not be sufficiently reduced to zero, probably due to the leakage of the *nisA* promoter in a high-copy system. Therefore, a conditional *rfbBD* mutant was constructed in which the transcription of the chromosomal *rfbBD* genes was placed under control of the *nisA* promoter, while the *rfbAC* genes remained under control of the original *rfb* promoter. For this purpose, the non-replicative plasmid pNZ4114 was transformed to strain NZ9000 resulting in integrants that contained the *rfbAC coding* sequences followed by the desired integration of the *tet* gene, the *Cm* gene derived termination sequence and the *nisA* promoter followed by the *rfbBD* coding sequences. One of the RfbB conditional mutants, strain NZ4114, was used for further analysis and found to be unable to grow in the absence of nisin in the medium, confirming that the *rfb* genes are essential for *L. lactis*.

To evaluate the effect of controlled *rfbBD* expression on growth, strain NZ4114 was grown overnight in medium containing different levels of nisin and was subsequently subcultured in medium lacking nisin. The optical density was followed in time (Fig. 5.4). These measurements revealed that addition of one ng ml^{-1} nisin leads to growth very similar to a wild-type NZ9000 culture. Furthermore, a stepwise lowering of the nisin concentrations in the overnight cultures coincides with a gradual reduction of the RfbBD production level and resulted in stepwise

reduction of growth rate and a lower final optical density. The sugar composition of the cell-wall polysaccharides (Table 5.2) produced by these cultures with limiting RfbBD production levels appeared not to be modified, indicating that sugar nucleotides are used preferentially for the formation of cell-wall sugars and are essential for cell growth, rather than for EPS production. In addition, these results indicate that the NICE system is a powerful tool for the production of conditional mutants in essential genes like the *rfbBD* genes. Moreover, this strategy allows the study of the phenotypic effects of a mutation in an essential gene (see below).

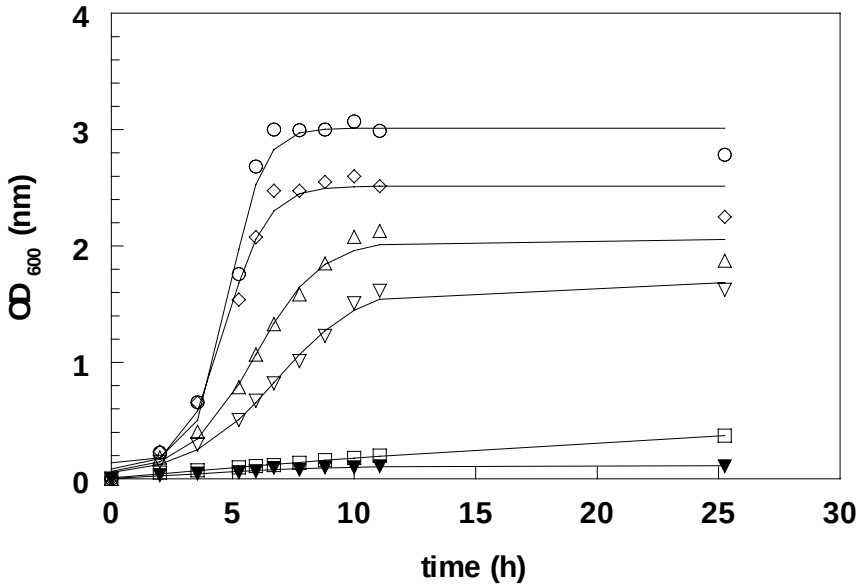

Fig. 5.4. Growth of *L. lactis* NZ4114; dependence on nisin addition. An overnight culture of NZ4114 grown in the presence of nisin concentrations of 1 (diamond), 0.5 (top triangles), 0.3 (down triangles), 0.05 (squares), and 0.01 (closed down triangles) ng ml^{-1} were subcultured in fresh medium without nisin and growth was followed during 25 h by OD_{600} measurement. Open circles represent a control culture grown in the presence of 1 ng ml^{-1} nisin. Data are representative of four independent experiments.

Table 5.2. Sugar compositions of the polysaccharides fraction of cell-walls of strain *L. lactis* NZ4114 and its parental strain grown in the presence or absence of nisin.

Strain	Nisin (ng ml^{-1})		Sugar composition (mol%)		
	Preculture	Subculture	Rhamnose	Galactose	Glucose
NZ9000	0	0	51.3 ± 0.1	15.5 ± 0.2	33.1 ± 0.0
NZ4114	1	1	53.5 ± 1.2	14.5 ± 1.3	32.4 ± 1.6
	1	0	54.1 ± 2.1	14.4 ± 2.3	31.9 ± 0.8
	0.5	0	48.1 ± 1.5	18.2 ± 0.2	34.7 ± 2.2
	0.3	0	48.2 ± 1.1	17.1 ± 0.0	34.7 ± 1.1
	0	0	NG[a]	NG	NG

[a]) NG, no growth.

To investigate the effect of limited *rfbBD* expression levels on EPS production, the EPS-producing capacity was introduced into strain NZ4114 by transformation with pNZ4030, which contains the NIZO B40 *eps* gene cluster. No differences in growth rate and EPS yield between the mutant strain and its parental strain were observed after 24 hours of fermentation when cells were grown in the presence of nisin (Table 5.3). However, EPS production by the mutant strain

was reduced to approximately 5% of that of the wild-type production level when grown under *rfbBD* limited expression conditions. These results primarily suggest that *rfb* expression is essential for both production of the NIZO B40 EPS and for growth. However, the observation that the final estimated cell number decreases stepwise with decreasing nisin concentration while EPS production levels are equal for all *rfbBD* limited strains, suggests that EPS production and growth are not coupled as previously suggested (Looijesteijn and Hugenholtz, 1999), and have different requirement for the precursor dTDP-rhamnose.

Table 5.3. EPS characteristics of *L. lactis* NZ4114 harboring the EPS-plasmid pNZ4030 grown in the presence or absence of nisin.

Strain	Nisin (ng ml^{-1})		EPS[a]			Molar mass[a]	R_g^{ac}	[η^d]
	pre	sub	(mg l^{-1})	(mg l^{-1} * OD_{600}^{-1})	(Glc:Gal:Rha)	(g mol^{-1}) * 10^6	(nm)	(m^3 kg^{-1}) * 10^{-3}
NZ9000	0	0	144 ± 1	55 ± 1	1 : 0.6 : 0.4	0.43 ± 0.01	44 ± 0.3	60 ± 1
NZ4114	1	1	114 ± 5	45 ± 1	1 : 0.6 : 0.4	0.42 ± 0.01	44 ± 0.0	58 ± 1
	1	0	7 ± 1	7 ± 1	1 : 0.3 : 0.2	1.7 ± 0.3	28 ± 0.6	5.2 ± 1
	0.5	0	6 ± 1	6 ± 1	1 : 0.4 : 0.2	1.9 ± 0.3	28 ± 0.7	3.6 ± 1
	0.3	0	8 ± 1	8 ± 1	1 : 0.3 : 0.1	1.2 ± 0.4	30 ± 0.2	3.2 ± 1
	0	0	NG[b]	NG	NG	NG	NG	NG

[a]) The values are averages based on at least two independent experiments.
[b]) NG, no growth.
[c]) R_g, radius of gyration.
[d]) η, viscosity.

Characterisation of EPS produced by the *rfb* mutant. The EPS produced by strain NZ4114 were analyzed by static light scatering (SLS) after size exclusion chromatography. Estimation of molar mass revealed that the EPS produced by strain NZ4114 had an average molar mass four-fold larger than that of wild-type NIZO B40 EPS (Table 5.3; Fig. 5.5A). Furthermore, SLS-measurements generated a two-fold smaller average radius of gyration (R_g) than that measured for NIZO B40 EPS (Fig. 5.5B). Moreover, the viscosifying properties of the mutant EPS in chemical defined medium, which can be calculated from the molar mass and the R_g (Tuinier *et al.*, 1999), was drastically decreased (Table 5.3). In addition, monomeric sugar analysis after hydrolysis of the purified EPS revealed that the EPS produced by the RfbBD conditional mutant strain consisted of the mono-saccharides glucose, galactose and rhamnose in the molar ratio of 1 : 0.3 : 0.2, which is clearly different from wild-type NIZO B40 EPS (1 : 1.7 : 0.8)(Table 5.3 and van Kranenburg *et al.*, 1997). These results indicate that the RfbBD conditional mutant produces EPS with an altered structure as the result of an at least partially altered repeating unit. Nevertheless, the polymerization and export machinery can still recognize and handle this altered repeating unit. Moreover, since the molar mass is increased the polymerization and export

machinery may even extend the polymer to greater length. Finally, the decreased radius of gyration points to a more compactly folded, less space-filling structure, which probably is the consequence of the altered primary structure.

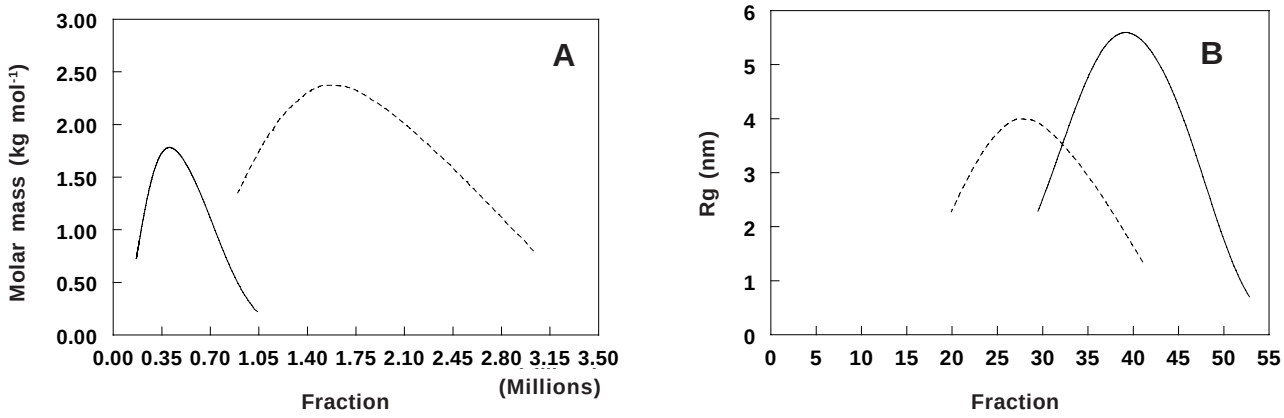

Fig. 5.5. The size distribution of molar mass (A) and radius of gyration (B) of NIZO B40 EPS (continuous line) and the altered EPS (dashed line).

Discussion

In this work we have described the identification and functional analysis of the chromosomal *L. lactis rfb* MG1363 operon that is involved in dTDP-rhamnose biosynthesis. Sequence analysis led to the identification of four *rfb* genes of which the predicted gene products display high levels of homology with proteins involved in the biosynthesis of dTDP-rhamnose. Evidence for the *rfb* functionality of the *L. lactis rfb* genes was obtained by overproduction of the Rfb proteins leading to increased Rfb activities. Moreover, expression of the *rfb* genes in *E. coli* complemented an *O*-antigen production mutation deleting the *S. flexneri* Rfb homologues.

Both in Gram-negative and Gram-positive bacteria the *rfb* genes are often genetically linked to genes involved in CPS or O-antigen production. Remarkably, even in *S. pneumoniae* serotypes that produce CPS that do not contain rhamnose, the *rfb* genes are linked to the *cps* locus (Muñoz *et al.*, 1997). In contrast, analysis of the chromosomal localization of the *rfb* genes in the *L. lactis* IL1403 genome sequence revealed that the lactococcal *rfb* gene cluster is not genetically linked to genes encoding related functions (Bolotin *et al.*, 2001). This resembles the situation in *S. mutans*, although in this and various other streptococci the *rfbD* ortholog was found distant from the *rfbA, rfbB,* and *rfbC* genes (Tsukioka *et al.*, 1997a; 1997b).

Functional overexpression of the *rfbACBD* or the *rfbAC* genes led to an increased level of Rfb proteins resulting in a two-fold increase in dTDP-rhamnose level. However, the increased Rfb enzyme activities did not result in production of more NIZO B40 EPS. Similarly, GalU

overproduction resulted in increased UDP-glucose and UDP-galactose levels but did not affect NIZO B40 EPS production (Boels *et al.*, 2001). These results suggest that the levels of individual sugar nucleotides do not control the EPS production level in *L. lactis* harboring pNZ4000 derivatives. This is in apparent contradiction to several reports that show that the activity level of enzymes involved in sugar nucleotide biosynthesis influence EPS production (Gilbert *et al.*, 2000; Levander *et al.*, 2002). However, this correlation seems to depend on the type of polysaccharide produced as was clearly shown for GalU activity in *L. lactis* (Boels *et al.*, 2001; Gilbert *et al.*, 2000). It is very well possible that a simultaneous increase of UDP-glucose, UDP-galactose and dTDP-rhamnose levels could positively affect the NIZO B40 EPS production level, since its repeating unit containes two glucose, two galatose and a rhamnose moiety. Alternatively, the level of NIZO B40 EPS production could also be controlled by the activity of the specific EPS biosynthesis machinery encoded by the EPS plasmid rather than by the level of sugar nucleotides. This is supported by the observation that overexpression of the priming glycosyltransferase *epsD* in *L. lactis* resulted in increased NIZO B40 EPS production levels (van Kranenburg *et al.*, 1997, 1999).

A conditional *rfbBD* mutant was constructed by introduction of the *nisA* promoter upstream of the *rfbB* gene in the chromosome of *L. lactis*. This mutant, *L. lactis* NZ4114, was not able to grow in the absence of nisin, indicating that the *rfbB* and/or *rfbD* genes are essential for *L. lactis*. This finding explains our lack of success in the construction of a *rfbB* mutant in *L. lactis* by using conventional knock-out strategies. In *L. lactis* NZ4114, lowering of the nisin concentration, resulted in reduction of growth rate and a lower final optical density. The sugar composition of the polysaccharide fraction of the cell-wall in this strain appeared unaffected. Remarkably, although rhamnose is a major component of cell polysaccharides in *L. lactis* as well as in *S. mutants* (Schleifer and Kilper-Bälz, 1987), inactivation of any of the four *S. mutans rml* genes led to viable cells lacking rhamnose in their cell-wall polysaccharide (Tsukioka *et al.*, 1997a; 1997b). We used the NICE system as a tool for the production of a conditional mutation in essential genes like the *rfbBD* genes, which allowed us to study the corresponding mutant phenotype. A similar strategy has recently been described for (F_0 F_1)-H^+-ATPase complex in *L. Lactis* (Koebmann *et al.*, 2000). However, since the NICE system can be implemented in many other hosts (Eichenbaum *et al.*, 1998; Kleerebezem *et al.*, 1997), this approach has potential to be used in other Gram-positive bacteria.

The EPS production level of the conditional *rfbBD* mutant *L. lactis* NZ4114 was only 5% of that of the wild-type EPS level when cells were grown under nisin limitation. This low level of production could be complemented by the addition of nisin to the medium. The effect of lack of *rfbBD* expression on both EPS production and growth in *L. lactis* can probably be explained by a crucial role for dTDP-rhamnose in cell-wall synthesis and an important role in the biosynthesis of the rhamnose-containing NIZO B40 EPS. The sugar composition of the EPS produced by strain NZ4114 consisted of glucose, galactose, and rhamnose in a ratio that is different from that of NIZO B40 EPS. These results indicate that the *rfbBD* conditional mutant produces EPS

with an altered composition as the result of an, at least partially, altered repeating unit. This implies that it might be possible to change the EPS composition by reducing the availability of EPS precursors via inactivation of specific precursor-forming enzymes. Although the yield is limited, the EPS polymerization and export machinery is still capable to recognize and process EPS with an altered repeating unit and is apparently not exclusively specific for a single repeating unit. This is corroborated by the finding that expression of the *S. thermophilus eps* gene cluster in *L. lactis* resulted in production of very low amounts of EPS with a repeating unit that differs from its native substrate due to the lack of lactococcal UDP-*N*-acetylglucosamine C4-epimerase activity leading to the incorporation of a galactose moiety instead of a GalNac moiety in the mutant EPS (Stingele *et al.*, 1999). However, it remains to be established what determines the low production level (6-10 mg l^{-1}) of the EPS produced by these lactococci.

The global sugar composition of the altered EPS produced by strain NZ4114 contains less rhamnose and galactose moieties and was shown to have a four-fold increased molecular mass compared to that of wild-type NIZO B40 EPS. Since the distribution of the molar mass of both the NIZO B40 and the altered EPS follow a typical symmetric Gaussian curve (Fig 5a), it can be concluded that the altered EPS are homogeneous polymers and do not represent a mixture of altered and native NIZO B40 polymers. Besides the four-fold increased molecular mass, the altered EPS had a two-fold decreased radius of gyration compared to that of wild-type NIZO B40 EPS. This finding suggests a drastic decrease of the viscosifying properties of these EPS compared to those of wild-type NIZO B40 EPS (Tuinier *et al.*, 2001). Moreover, the decreased radius of gyration indicates that the folding of the backbone of the altered EPS is much less hindered by side chains, resulting in higher chain flexibility and a more compactly folded structure. Hence, it is likely that the increase of molar mass of the altered EPS is due to an increased chain length rather than an increase in the number or size of the side chains. Furthermore, these results suggest that the repeating unit of the altered EPS is a modification of that of the NIZO B40 EPS and is partially lacking its side chains consisting of rhamose and galactose-phosphate. Finally, the production by strain NZ4114 of a longer and more compact EPS than the native EPS suggests that the chain length determination mechanism is dependent on the three-dimensional conformation of the polymer rather than on only the length of the chain itself.

Evaluation of the dTDP-rhamnose biosynthesis pathway described here allowed an assessment of the role of the *rfbACBD* genes in *L. lactis* using overexpression and disruption studies of these genes. We could significantly influence the dTDP-rhamnose level, which is a precursor for cell-wall polysaccharides as well as for EPS biosynthesis in *L. lactis*. We were also able to influence EPS production levels and even its repeating unit sugar composition by using a conditional *rfbBD* mutant. Various reports have previously established the possibilities to modulate polysaccharide biosynthesis by engineering at the level of specific *eps* genes (for review see van Kranenburg *et al.*, 1999). To our knowledge this is the first report showing that modulation of the household enzyme levels could lead to EPS production with an altered

composition. These results enlarge the knowledge base required for efficient targeting of bottlenecks in EPS biosynthesis and provide new opportunities for creating structural diversity of polysaccharides with novel properties.

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Chapter 6

Chapter

Increased Exopolysaccharide Production in *Lactococcus lactis* by Increased Expression Levels of the NIZO B40 *eps* Gene Cluster

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Abstract

Exopolysaccharides (EPS) play an important role in the rheology and texture of fermented food products. The EPS production capacity of *Lactococcus lactis* strain NIZO B40 is encoded on the Eps-plasmid pNZ4000. We varied the expression levels of the NIZO B40 *eps* genes in the plasmid-free *L. lactis* strain MG1363 to investigate their role in control of the production level of NIZO B40 EPS biosynthesis in *L. lactis*. In a primary approach, the replication gene, *repB2*, of the pNZ4000-derivative pNZ4030 was overexpressed, resulting in a two-fold increased Eps-plasmid copy number and a 35% increased NIZO B40 EPS production level compared to that of the wild-type. In a second strategy the entire NIZO B40 *eps* gene cluster was cloned on a high copy vector. This approach led to a nine-fold increased Eps-plasmid copy number, and consequently an almost three-fold increased *eps* expression level resulting in a four-fold increased NIZO B40 EPS production level. The kinetic viscosity of M17 grown cultures were 1.6-fold increased compared to wild-type cells, indicating that the increased EPS production levels positively influenced the viscosity of the M17 medium.

Introduction

Microbial exopolysaccharides (EPS) are widely applied as thickening, gelling and stabilizing ingredients in the food industry. The commercially most important microbial EPS is xanthan gum, a complex polymer that is produced by the phytopathogen *Xanthomonas campestris* (Sutherland, 1998; Becker *et al.*, 1998). In the dairy industry EPS-producing lactic acid bacteria (LAB) are used to improve the texture of fermented diary products such as yogurt. Moreover, it has been suggested that EPS produced by LAB confer health benefits (Hosono *et al.*, 1997; Kitazawa et al, 1991; Nakajima *et al.*, 1992). Different LAB produce a wide variety of structurally different polymers, which have potential applications as food additives. These polysaccharides would be preferable over polymers such as xanthan since their production hosts have a food-grade status, based on their long-term application in food fermentations.

The EPS production capacity was found to be encoded on the chromosome for thermophilic yogurt bacteria (Lamothe, 2000; Almiron-Roig *et al.*, 1996, Stingele *et al.*, 1996), while it appeared to be plasmid located in *Lactococcus lactis* (van Kranenburg *et al.*, 1997, 1998). In all cases genes involved in EPS biosynthesis are organized in gene clusters that display significant conservation in both organization and sequence. *L. lactis* strains are known to produce various types of EPS (van Casteren, 2000; van Kranenburg *et al.*, 1999) which have different viscosifying properties (Tuinier et al, 2001). Among those, the best characterized is strain NIZO B40, which harbors a 42,180-bp EPS-plasmid, pNZ4000, containing the 12-kb *epsRXABCDEFGHIJKL* operon (van Kranenburg *et al.*, 1997, 2000). This strain produces a polymer with a regular repeating unit, →4)[α-L-Rha*p*-(1→2)][α-D-Gal*p*-1-PO_4-3]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→. Functional analysis of the glycosyltransferase genes established that biosynthesis of the repeating unit backbone is initiated by the linkage of glucose derived from UDP-glucose to a lipid carrier by the priming glucosyltransferase EpsD. Subsequently the combined activity of EpsE and EpsF link a second glucose to this lipid-linked glucose thereby forming a lipid-linked cellobiose. Finally, EpsG links galactose to the lipid-linked cellobiose, completing the NIZO B40 repeating unit backbone structure. Subsequent steps of the repeating unit synthesis most likely involve the addition of the rhamnose and the galactose-phosphate to the galactose moiety in the backbone, which has been suggested to be catalyzed by EpsH and EpsJ, respectively (van Kranenburg *et al.*, 1999).

EPS are only formed at relatively low production levels that generally vary from with 40-800 mg l^{-1}, compared to *X. campestris,* which produce 10-25 g xanthan l^{-1} (Becker *et al.*, 1998). However, some EPS from LAB are very effective biothickeners when produced *in situ* (Hess *et al.*, 1997; Tuinier *et al.*, 2001). The differences between polymers can be explained by their intrinsic viscosity, in which the molar mass and the radius of gyration (a measure for size of a polymer in solution) were found to be the most important parameters (Tuinier *et al.*, 2001). Among several EPS, the NIZO B40 EPS appears to have useful molecular properties which is reflected

by the desirable rheological properties of the products produced with this strain (Ruas-Madiedo *et al.*, 2002). Studies aimed at engineering EPS structure hold promise for the directed engineering of rheological properties of EPS. A crucial factor in the design of these modified EPS is knowledge of the physical properties of the EPS and its interactions with other components in the product. Unfortunately, the modified LAB polymers to date are only formed at approximately ten-fold of that produced by the wild-type strains (Boels *et al.*, 2002; Stingele *et al.*, 1999). Therefore, bottlenecks in EPS production have to be identified before modified EPS could be of economical interest for the use as *in situ* produced food ingredients.

To date, no reports have appeared on regulation of lactococcal EPS production by environmental or endogenous factors. Previously, we have demonstrated the overproduction of the NIZO B40 priming glucosyltransferase, EpsD, in a strain lacking *epsD* in its *eps* gene cluster, resulted in an increased EPS production compared to that of the control strain harboring a plasmid with the intact *eps* gene cluster (van Kranenburg *et al.*, 1999). These data suggest that elevation of the level of *eps* gene expression could result in higher EPS production. An increase of *eps* gene expression may be achieved by elevating the copy number of the Eps-plasmid. The NIZO B40 Eps-plasmid pNZ4000 contains four highly conserved replication regions with homologous *rep* genes, *repB1*, *repB2*, *repB3*, and *repB4* that belong to the lactococcal theta replicon family (van Kranenburg *et al.*, 2000; Frère *et al.*, 1995). Theta-replicating lactococcal plasmids generally have low copy numbers but show high stability (Jannière *et al.*, 1993). Each replicon encodes a replication protein (RepB) that is likely to autoregulates its own production by binding to the *repB* promoter region (Brandl *et al.*, 1993; Swinfield *et al.*, 1990). Therefore, increased RepB production may have an effect on the copy number of the Eps-plasmid, which could result in higher EPS production levels.

Here we present two strategies to increase the copy-number of the NIZO B40 *eps* gene cluster. One included the *in trans* overexpression of the *repB2* gene of pNZ4000. The other included the overexpression of the complete NIZO B40 *eps* gene cluster, by cloning it on a relatively high copy vector pIL253. Both strategies led to elevated *eps* transcription levels and subsequently to increased EPS production.

Materials and methods

Bacterial strains, plasmids, and media. *L. lactis cells* were grown without aeration at 30°C in M17 broth (Merck, Germany), in a chemically defined medium (CDM) (Looijesteijn and Hugenholtz, 1999) supplemented with 2 % (wt/vol) glucose, or in HP milk (Van Marle and Zoon, 1995) supplemented with 2 % (wt/vol) glucose and 0.5 % (wt/vol) yeast extract (Difco, The Netherlands). When appropriate, the media contained chloramphenicol (10 $\mu g\ ml^{-1}$), erythromycin (10 $\mu g\ ml^{-1}$), tetracycline (2.5 $\mu g\ ml^{-1}$), or ampicillin (100 $\mu g\ ml^{-1}$).

DNA techniques. Small-scale isolation of *E. coli* plasmid DNA was performed as described by Sambrook *et al.* (1989). Isolation and transformation of *L. lactis* DNA were performed as previously described (de Vos *et al.*, 1989). For diagnostic analysis total DNA was isolated from exponentially grown lactococcal cells (20 µl) that were harvested by centrifugation and disrupted by a microwave treatment (2 min.; 800 W). Disrupted cell pellet, including total DNA, was suspended in 20 µl water and directly used for PCR reactions (see below). Isolation of RNA was performed with help of a RNeasy kit (Qiagen, The Netherlands) by following the instructions of the manufacturer. Reverse transcriptase reactions were performed using Omniscript reverse transcriptase (Qiagen, The Netherlands) following the instructions of the manufacturer. The RT-primers (Table 6.1) used contained a tag-extension for RNA and DNA differentiation in subsequent real-time PCR analysis (Sybesma *et al.*, 2001).

Oligo name	Oligo sequence
DNA linker	
link-F	5'-TCGAGCATGCCATGGCATGG-3'
link-R	5'-GATCCCATGCCATGGCATGC-3'
DNA primers	
TM-epsC-F	5'-CATCTTAAATGCACGTGACGT-3'
TM-epsC-R	5'-AGTGTCACTGGTCATTTTGG-3'
TM-epsC-R2	5'-ACTTTCATGGATTTGGAAGTGTC-3'
TM-pepN-F	5'-TTGGCACACAGTTTGAAAGCC-3'
TM-pepN-R	5'-CAAATCGAAAGTTGCTTTCGC-3'
TM-pepN-R2	5'-CACTATGGCTAACCGTTAATCG-3'
TM-ery-F	5'-TTCACCGAACACTAGGGTTGC-3'
TM-ery-R	5'-CATTCCGCTGGCAGCTTAAG-3'
TM-ery-R2	5'-TCGTCGCATGGAGCATTCC-3'
Probes	
TM-epsC-FAM	5'-FAM-CGCATCTGATGCAACAAAATGCGTA-TAMRA-3'
TM-pepN-FAM	5'-FAM-TTTTGCTCGCCAAGCTTTCCCATCT-TAMRA-3'
TM-ery-FAM	5'-FAM-TGCACACTCAAGTCTCGATTCAGCA-TAMRA-3'
RT PCR primers	
TM-pepN-RT	5'-CACTATGGCTAACCGTTAATCGAAAGTTGC-3'
TM- ery-RT	5'-TCGTCGCATGGAGCATTCCGCTGG-3'
TM-epsC-RT	5'-ACTTTCATGGATTTGGAAGTGTCACTGGTC-3'

Table 6.1. Oligo nucleotide sequences of a DNA linker, forward (F), reverse (R) primers used in real-time PCR, and reverse transcriptase (RT) primers used in RT PCR.

Construction of plasmids. To overproduce the replication protein, RepB2, of pNZ4000, the *repB2* gene (van Kranenburg and de Vos, 1998) was cloned from pNZ4030 as a 1.6-kb *Cla*I-*Eco*RI fragment (with the *Cla*I site blunted using Klenow) in pNZ8020 (de Ruyter *et al.*, 1996), which was digested with *Sma*I-*Eco*RI, The resulting plasmid pNZ4119 was transformed to strain NZ9000 harboring pNZ4030 (van Kranenburg *et al.*, 1997).

The *eps* overexpression plasmid was constructed by first inserting a linker which consisted of a double stranded oligonucleotide (link-F and link-R; Table 6.1) containing a *Nco*I site, into pIL253 (Simon and Chopin, 1988) digested with *Xho*I and *Bam*HI, to obtain a convenient cloning site. Subsequently, the tetracycline-resistant pNZ4000-derivative pNZ4130 (Boels *et al.*, 2001) was digested with *Nco*I resulting in a 17-kb *Nco*I fragment from pNZ4130 containing the entire NIZO B40 *eps* gene cluster and a 25-kb fragment. Both fragments were ligated to similarly digested pIL253 containing the *Nco*I site, using shotgun cloning, and transformed to *L. lactis* NZ9000 (Kuipers *et al.*, 1998). Transformants were screened on erythromycin-resistance and ropyness and one colony was selected, which contained the anticipated Eps-plasmid pNZ4120.

Real-time PCR. Primer pairs and probes (Table 6.1) were designed using PrimerExpress software (Applied Biosystems, Germany). Primer pairs were selected, having similar predicted melting temperature (55-60°C), and purchased from Pharmacia. The TaqMan™ probes have a higher melting temperature (65-68°C) compared to the primers and contained a FAM reporter dye connected to the 5' end, and a TAMRA quencher dye to the 3' end, and were purchased from Applied Biosystems. To detect the amount of plasmid DNA we designed two probes, TM-epsC-FAM and TM-ery-FAM, which could hybridize with the plasmid located *epsC* and erythromycin (*ery*) genes, respectively. In analogy, a pepN-probe, which could hybridize on the chromosomal DNA encoded *pepN* gene, was used as an internal standard of chromosomal DNA quantification. To generate a PCR product on DNA template the primer pairs on *epsC* (TM-epsC-F and TM-epsC-R), *pepN* (TM-pepN-F and TM-pepN-R) and *ery* (TM-ery-F and TM-ery-R) were used. To generate a PCR product on DNA-RNA hybrid template the primer pairs on *epsC* (TM-epsC-F and TM-epsC-R2), *pepN* (TM-pepN-F and TM-pepN-R2) and *ery* (TM-ery-F and TM-ery-R2) were used. The reverse primers used were designed in a way that they only hybridized at the tag of the RT-primers to be able to differentiate between true cDNA products and chromosomal DNA-based, false positive reaction products (Sybesma *et al.*, 2001). PCR was carried out using the ABI Prism™ 7700 sequence detection system and the TaqMan core reagent kit, 96-well MicroAmp™ optical plates and caps (All from Applied Biosystems, The Netherlands) in reaction volumes of 50 µl, according to the recommendations of the manufacturer. PCR reactions comprised 200 nM forward primer, 200 nM reverse primer, 100 nM probe, and 10 µl total DNA solution. PCR conditions were 2 min. 50°C, 10 min. 95°C, followed by 40 cycles of 10 sec., 95°C; 1 min., 60°C. Data evaluation was carried out using the ABI Prism sequence detection software. The threshold cycle number (C_t) was determined as the number of cycles at which the fixed threshold fluorescence emission (0.3 relative quantity) was detected (Higuchi *et al.*, 1993). Copy number values were calculated as relative copy numbers ($N_{relative}$) with the formula:

$$N_{relative} = 2^{(Ct,\ gene\ X\ -\ Ct,\ pepN)}$$

using the C_t value of the chromosomal encoded *pepN* gene as the internal standard.

EPS analysis. EPS was isolated from CDM according to Looijesteijn and Hugenholtz (1999). EPS was isolated from fermented milk according to the method described by van Marle and Zoon (1995). EPS quantification and characterization was performed as described by Looijesteijn and Hugenholtz (1999).

Viscosity measurements. The flow time of M17 grown cultures, was measured with an Ubbelohde viscometer with a capillary of diameter 0.63 mm as described by van Marle and Zoon (1995). The measurements were performed at 20°C ± 0.5. The viscosity of fermented milk was measured using a Posthumus funnel as described by van Marle and Zoon (1995). A funnel with a diameter opening size of 8 mm was filled with stirred fermented milk and the time (s) taken to pass the mark inside the funnel was used as a measure of apparent viscosity of the samples. Measurements were carried out at 20°C ± 0.5.

Results

Effect of RepB2 overproduction on EPS production. A primary approach to elevate Eps-plasmid copy number was taken by overproduction of the replication protein RepB. Although there are four active replicons present on Eps-plasmid pNZ4000, the *repB2* gene is the only replication gene on its erythromycin-resistant-derivative pNZ4030. Therefore, plasmid pNZ4119 carrying the *repB2* gene including its own promoter was introduced into strain NZ9000 harboring pNZ4030 (van Kranenburg *et al.*, 1997). EPS production was determined in triplicate for cultures of *L. lactis* NZ9000 cells harboring pNZ4030 or both pNZ4030 and pNZ4119 (Table 6.2). The EPS production level of cell cultures overexpressing *repB2* increased with 35%, confirming that the level of *repB2* expression influences the production level of EPS.

Table 6.2. EPS production, DNA copy numbers of the NIZO B40 Eps-plasmid and expression levels of the *eps* genes of strain *L. lactis* NZ9000 harboring pNZ4030 and pNZ8048 and of NZ9000 harboring pNZ4119. Copy numbers and expression levels were determined relative to the chromosomal *pepN* gene.

Strain (plasmid)	EPS[a]		Relative DNA copy number[a]		Relative transcription level[a]
	(mg l^{-1})	(mg l^{-1} * OD_{600}^{-1})	Ery-probe	EpsC-probe	
NZ9000 (pNZ8048)	104 ± 5	41 ± 1	2.6 ± 0.4	2.5 ± 0.4	0.09 ± 0.02
NZ9000 (pNZ4119)	140 ± 4	55 ± 1	5.5 ± 3.4	4.0 ± 0.7	0.20 ± 0.12

[a]) The values are averages based on at least three independent experiments.

To evaluate whether the increased EPS production was the result of an increased EPS-plasmid copy number, the copy number of the Eps-plasmid DNA relative to chromosomal DNA was determined using real-time PCR. The thresholds' cycle number (C_t) was determined and used to calculate the relative gene copy number for each strain (Table 6.2). In the strain harboring both pNZ4030 and pNZ4119, an increase of relative copy number of almost two compared to the strain harboring pNZ4030 alone was achieved, indicating that the RepB2 protein promotes the Eps-plasmid copy number.

To analyze the effect of increased Eps-plasmid copy number on the expression of the *eps* genes, we quantified mRNA isolated from strain NZ9000 harboring pNZ4119 and pNZ4030. The mRNA isolated from exponentially grown cells was reverse transcribed using RT primers that contained a dedicated 5'-tag for cDNA/DNA discrimination (Sybesma *et al.*, 2001), designed on the 3' end of the plasmid located *epsC* and *em* genes and the chromosomal located *pepN* gene. The cDNA generated was subsequently amplified, detected and quantified in real-time PCR using primers pairs, in which the reverse primer was designed on the tag of the RT-primer, and the relative gene expression levels were calculated on basis C_t values obtained for these real-time PCR reactions (Table 6.2). A more than two-fold increase of relative *eps*-gene expression level was achieved using the RepB overproduction strategy.

Taken together, overexpression of the *repB* gene significantly increased the EPS production level, which is mediated by the increased Eps-plasmid copy number and the subsequent increased *eps* gene expression levels.

Cloning of the NIZO B40 *eps* gene cluster on a high copy vector. To evaluate whether we could further increase the copy number of the NIZO B40 EPS-plasmid, we cloned the entire 17-kb NIZO B40 *eps* gene cluster on the high copy vector pIL253. Subsequently, pIL253 derivative pNZ4120 harboring the entire NIZO B40 *eps* gene cluster including its own promoter was transformed to strain NZ9000. The resulting strain was grown to exponential phase, and DNA and RNA were isolated. The relative copy number of pNZ4120 DNA to chromosomal DNA was analyzed using real-time PCR, by determination of the initial amount of plasmid located *epsC* and *em* DNA template relatively to the chromosomal located *pepN* DNA template (Table 6.3).

Table 6.3. Relative DNA copy numbers of the NIZO B40 Eps-plasmid derivatives and relative expression levels of the *eps* genes in strain *L. lactis* NZ9000 harboring pNZ4030 or pNZ4120. Copy numbers and expression levels were determined relative to the chromosomal *pepN* gene.

Strain (plasmid)	Relative DNA copy number[a]		Relative transcription level[a]
	Ery-probe	EpsC-probe	
NZ9000 (pNZ4030)	2.2 ± 1.6	1.3 ± 0.1	0.07 ± 0.03
NZ9000 (pNZ4120)	16 ± 2.1	13 ± 1.8	0.18 ± 0.01

[a]) The values are averages based on at least three independent experiments.

Almost nine-fold higher relative copy number was obtained in cells of strain NZ9000 harboring pNZ4120 relative to those harboring pNZ4030, indicating that EPS plasmid copy number could be increased significantly by replacing its endogenous replication machinery by that of pIL253.

To investigate the effect of the nine-fold increased EPS-plasmid copy number on the expression of the *eps* genes we quantified the *eps*-mRNA level of strain NZ9000 harboring pNZ4120 using the same PCR based strategy as was described above for the RepB overproduction approach (Table 6.3). The relative *eps* gene expression level was almost three-fold increased relative to pNZ4030. These results further establish that the expression of the *eps* genes can be raised to a higher level by increasing the copy number of the Eps-plasmid. Almost four-fold increased level of EPS production was achieved of cells harboring pNZ4120 compared that of cells harboring pNZ4030 (Table 6.4), indicating that *eps* gene expression plays an important role in control of EPS production in *L. lactis*.

Table 6.4. EPS production and kinetic viscosity determined by Ubbelohde experiments of *L. lactis* NZ9000 derivatives harboring NIZO B40 EPS-plasmid derivatives grown in CDM.

Strain (plasmid)	EPS[a]		Kinetic viscosity[b]
	(mg l^{-1})	(mg l^{-1} * OD_{600}^{-1})	(m^2 s^{-1}) * 10^6
NZ9000 (pNZ4030)	93 ± 7	35 ± 3	1.4 ± 0.1
NZ9000 (pNZ4120)	343 ± 5	128 ± 4	2.2 ± 0.1

[a]) The values are averages based on at least three independent experiments.

[b]) The values are averages based on at least two independent experiments. The kinetic viscosity of medium is 1.2 *10^6 m^2 s^{-1}.

Overexpression of the *eps* gene cluster resulted in a reduced growth rate compared to that of its parental strain as was also observed in the RepB2 overproducing strain (data not shown). In addition, the relative carbon flux towards EPS production was three-fold increased while the relative biomass formation remained the same (Table 6.5). These results suggest that increased EPS production lead to a metabolic burden due to the production of the sugar nucleotides, which are required for both EPS production and growth. This suggestion is supported by the observation that sugar-nucleotide pools in EPS producing cells are lower compared to those of non-EPS-producing cells (Looijesteijn *et al.*, 1999; Ramos *et al.*, 2001) and the finding that the lactate formation was significantly reduced due to increased EPS formation and slightly increased acetate and ethanol formation. These results indicate that an increase of EPS production may cause a decrease of glycolytic flux, which subsequently may be reflected in the reduced growth rates.

Effect of increased NIZO B40 EPS production levels on viscosity. The effect of increased EPS production levels on biophysical characteristics of fully grown cultures was analyzed in

Table 6.5. Carbon balance of glucose consumption and product formation in strain *L. lactis* NZ9000 harboring Eps-plasmid pNZ4030 or pNZ4120. In brackets the percentages towards glucose consumption are given.

Strain (plasmid)	Glucose consumption (C-mol)	Carbon formation (C-mol)					Recovery (%)
		Biomass	EPS	Lactate	Acetate	Ethanol	
NZ9000 (pNZ4030)	35.8 ± 0.09	0.071 ± 0.004 (0.19%)	2.28 ± 0.07 (6.5%)	30.6 ± 0.02 (85%)	0.21 ± 0.01 (0.6%)	0.03 ± 0.02 (0.04%)	92 ± 0.6
NZ9000 (pNZ4120)	35.1 ± 0.04	0.064 ± 0.001 (0.19%)	7.27 ± 0.44 (20%)	27.3 ± 0.28 (85%)	0.33 ± 0.04 (0.9%)	0.33 ± 0.06 (0.9%)	100 ± 2.2

strain NZ9000 harboring pNZ4120. The biophysical parameters including molar mass and the radius of gyration of the EPS produced by the overproducing strain were not significantly different from the EPS produced by strain NZ9000 harboring pNZ4030 (data not shown). Apparently, the copy number does not influence the type or average chain length of the EPS, although it can influence the production level. Furthermore, the viscosity of the cultures was analyzed using Ubbelohde analysis. The kinetic viscosity of the overproducing strain was 1.6-fold higher compared to wild-type cells grown in M17 medium, indicating that increased EPS production levels positively influenced the viscosity of the M17 medium. However, when the mutant strains were grown in HP-milk no increase of viscosity of mutant cultures was measured by using Posthumus funnel analysis, although the EPS production levels were increased (Table 6.6). Remarkably, the highest viscosity was measured for the wild-type strain. Due to the overall lower level of EPS concentration in the HP-milk compared to M17 fermented cultures the viscosifying enhancing effect in fermented milk may be reduced. Moreover, the acidification rate in the early and the late logaritmic growth phase was lower of cells of strain NZ9000 harboring pNZ4120 compared to those harboring pNZ4130 (data not shown), suggesting that pH and growth besides EPS concentration influence the biophysical characteristics of the fermented milk product.

Table 6.6. Apparent viscosity in Posthumus seconds of stirred *L. lactis* NZ9000 harboring pNZ4030 or pNZ4120 grown in HP-milk.

Strain (plasmid)	EPS (mg l^{-1})	Posthumus (s)
NZ9000 (pNZ4030)	12	146 ± 6
NZ9000 (pNZ4120)	91	84 ± 1

Discussion

EPS produced by LAB contribute to the rheology and texture of fermented products and are therefore of interest for food application as natural biothickeners. However, EPS production levels of LAB are limited. To be able to increase EPS production levels we have studied the well-characterized NIZO B40 EPS biosynthesis in *L. lactis*. EPS biosynthesis is dependent on specific *eps* genes located on the Eps-plasmid, pNZ4000, and on several household genes, which are required for the formation of EPS precursor molecules, the sugar nucleotides. The results indicate that the efficiency of the EPS production by *L. lactis* NIZO B40 can be improved significantly by increasing the expression of the *eps* genes.

A *gusA*-gene fusion to the promoter of the *eps* gene cluster has been used to study its expression (Looijesteijn and Hugenholtz, 1999; van Kranenburg, 1999). The results suggest that the NIZO B40 *eps* genes are constitutively expressed and do not appear to be subjected to regulation by environmental factors. However, the first gene of the NIZO B40 *eps* gene cluster, *epsR*, encodes a putative protein which is homologous to regulator proteins containing a DNA-binding domain (van Kranenburg *et al.*, 1997). Moreover, the last gene of the cluster, *orfY*, located downstream and in opposite direction of the *eps* gene cluster, encodes a putative protein with homology to LytR, a transcription attenuation protein of the *Bacillus subtilis lytABC* and *lytR* operons (van Kranenburg *et al.*, 1997; Lazarevic *et al.*, 1992). For gene clusters involved in capsule synthesis in *S. pneumoniae* and group B streptococci, or in EPS biosynthesis in *S. thermophilus,* the first gene is believed to be involved in regulation as it encodes a protein homologous to LytR (Joly and Stingele, 2001; Guidolin *et al.*, 1994; Kolkman *et al.*, 1997, Koskiniemi *et al.*, 1998, Muñoz *et al.*, 1997, Stingele *et al.*, 1996). Both *epsR* and *orfY* seem to be conserved in lactococcal *eps* gene clusters (van Kranenburg *et al.*, 1999). However, their role in regulation of *eps* gene expression, if any, remains to be established. Recently, a relationship between tyrosine kinase phosphorylation and polysaccharide synthesis has been reported (Cartee *et al.*, 2002; Morona *et al.*, 2000; Niemayer and Becker, 2001; Vincent *et al.*, 2000; Wugeditsch *et al.*, 2000). In pneumococci CpsC is required for initial phosphorylation of CpsD, that subsequently regulates capsule synthesis (Cartee *et al.*, 2002; Morona *et al.*, 2000). It is feasible that similar regulation is mediated by the gene products of *L. lactis epsB* and *epsA*, which are the *cpsC* and *cpsD* analogues, respectively.

In a first approach to increase the copy number of the Eps-plasmid we overproduced the replication protein RepB2 of the NIZO B40 Eps-plasmid pNZ4030 and in a second approach we cloned the entire NIZO B40 *eps* gene cluster on a high copy plasmid. We evaluated whether both strategies resulted in an increased copy number of the *eps* gene cluster. The most commonly used method for determining plasmid copy number employs the comparison of the quantity of plasmid DNA to the quantity of hosts chromosomal DNA. However, plasmid and mRNA isolation from EPS producing cells is hampered by the fact that EPS production disturbs efficient harvesting

of the cells. To circumvent these limitations, we used a quantitative real-time PCR in combination with diagnostic sampling approach (Ginzinger *et al.*, 2000). The relative Eps-plasmid copy number was almost two-fold increased in the RepB-overproducing strain NZ9000 harboring pNZ4119 compared to that of its parental strain. In analogy, the relative EPS-plasmid copy number of strain NZ9000 harboring pNZ4120 was over nine-fold increased. These results seem to support the hypothesis that the RepB protein autoregulates its own production and, consequently, the plasmid copy number. Furthermore, the relative expression levels of the *eps* genes in the RepB2 overproducing strain and strain NZ9000 harboring pNZ4120 were significantly increased two-fold and almost three-fold, respectively, compared to that of their parental strains. A similar increase in relative expression was established found for the expression of the erythromycin gene (data not shown). These results indicate that both strategies led to increased *eps* gene expression levels, but the latter is more efficient.

Previously, we reported that by increasing the enzyme activity levels of the household genes involved in the EPS biosynthesis pathway led to increased EPS precursor levels (Boels *et al.*, 2001; Boels *et al.*, 2002). However, this did not result in increased NIZO B40 EPS production levels. In contrast, EPS analysis confirmed that the level of *repB2* expression slightly increased the production level of EPS. Remarkably, the EPS production was even four-fold elevated by cloning of the NIZO B40 *eps* gene cluster on a high-copy vector. Since in both approaches the relative expression level of the *eps* genes was increased, these results imply that the elevated EPS production level was directly caused through the increased expression level of the NIZO B40 *eps* gene cluster. Moreover, these results even suggest a correlation of *eps* gene expression levels and the EPS production level, which may indicate that the production level can even be more elevated. Taken together, these results indicate that the maximal EPS production level is limited by the activity level of the expression products of the *eps* gene cluster, rather than by the level of sugar nucleotides. However, this hypothesis might not hold true for the biosynthesis of EPS other than NIZO B40. The latter is supported by the findings that the level of *S. pneumoniae* type 3 CPS production in *L. lactis* can be dramatically increased by the expression of a pneumococcal CPS precursor forming enzyme GalU (UDP-glucose-pyrophosphorylase) (Gilbert *et al.*, 2000).

Among several lactococcal EPS NIZO B40 EPS showed the largest viscosifying effect in fermented milk due to the stiffness of the EPS molecule which probably originates both from a stiff backbone and steric hindrance of the side groups (Ruas-Madiedo *et al.*, 2002). These biophysical parameters make NIZO B40 an interesting polymer for application. Indeed the kinetic viscosity of cells of strain NZ9000 harboring pNZ4120 grown in M17 medium was significantly influenced when four-fold more EPS was produced. Although, the viscosity of fermented milk products is dependent on the protein network (van Marle and Zoon, 1995) and the EPS concentration (Sebastiani and Zelger, 1998), the induction of the EPS-production level in this strain did not affect the viscosity of fermented milk. Apparently, the increase of NIZO B40 EPS concentration was too low to show a significant effect on the viscosity of our milk grown cultures.

This contrasting effect in viscosity might be caused by the different acidification profile, which is likely the result of the significant decreased lactate production of strain NZ9000 harboring pNZ4120 compared to that of strain NZ9000 harboring pNZ4030. In addition, it is observed that the contribution of EPS to the properties of food products not only depends on the properties of the EPS itself but also on the interactions with various components in food products like proteins (van Marle and Zoon, 1995).

Evaluation of the EPS biosynthesis model described here allowed us to target an important bottleneck in EPS production. We could increase the NIZO B40 EPS production level four-fold by overexpression of the NIZO B40 *eps* genes in *L. lactis*. In addition, the results predicted that there might be a direct correlation between *eps* gene expression levels and EPS production level, suggesting that the EPS production level might be increased further. The identification of EPS production bottlenecks is important for future challenges for the construction of lactococcal strains that produce EPS with novel properties. In addition, these results are the first steps towards developing *L. lactis* into a production host of EPS for the application as food additives.

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Chapter 7

Chapter

Summary and Concluding Remarks

This thesis describes the genetics of exopolysaccharide (EPS) biosynthesis in *Lactococcus lactis*. Metabolic engineering approaches were used to generate insight in the EPS biosynthesis model (Fig. 7.1) by overproduction or depletion of individual groups of enzymes involved.

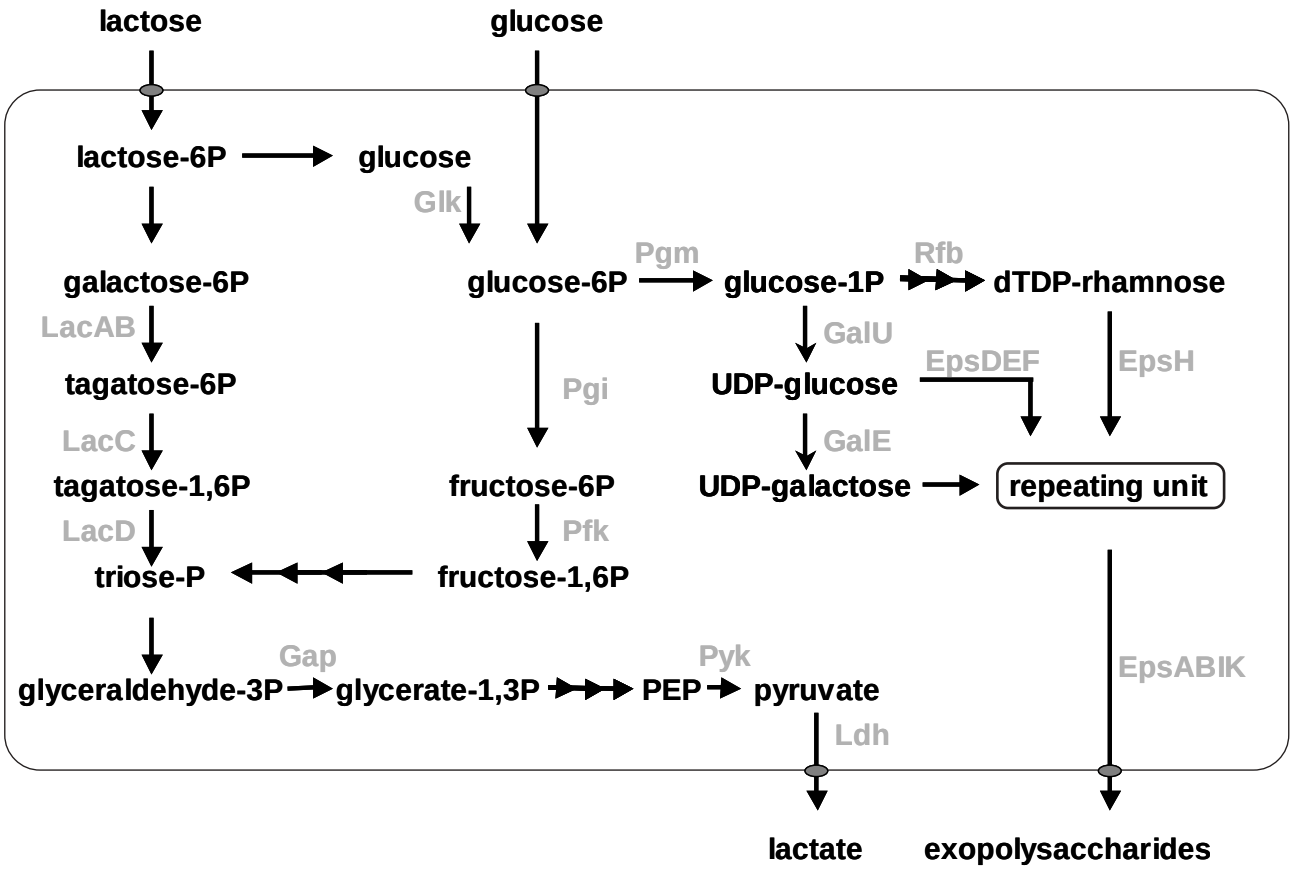

Fig. 7.1. Schematic representation of pathways involved in sugar fermentation via glycolysis to lactate and/or other acids and biosynthesis of exopolysaccharides (EPS). The numbers indicated in superscript point to the chapters in this thesis in which a more detailed study is described. Enzymes involved: Glk, glucokinase; Pgi, phosphogluco isomerase; Pfk, phophofructokinase; Fbp, frucosebiphosphatase; Gap, glyceraldehyde-3-phosphate dehydrogenase; LacABCD, tagatose-6-P pathway; Pgm, phosphoglucomutase; GalU, UDP-glucose pyrophosphorylase; GalE, UDP-galactose epimerase; Rfb: dTDP-rhamnose biosynthetic system consisting of RfbA, glucose-1P thymidylyltransferase; RfbB, dTDP-glucose-4,6 dehydratase; RfbC, dTDP-4-keto-6-deoxy-*D*-glucose-3,5 epimerase; and RfbD, dTDP-4-keto-*L*-rhamnose reductase; EpsDEFG, NIZO B40 EPS repeating unit backbone glycosyltranferase machinery; EpsHJ, putative NIZO B40 repeating unit side-chain glycosyltransferase machinery; EpsABIK, NIZO B40 polymerization and export machinery.

EPS biosynthesis

Lactic acid bacteria (LAB) have a long history of safe use in the production of fermented foods. EPS produced by LAB play an important role in the rheological properties and the texture of fermented milk products. *L. lactis* strains are known to produce various types of EPS (van Casteren, 2000; van Kranenburg *et al.*, 1999a), which have different viscosifying properties (Tuinier *et al.*, 2001). As compared to other EPS, the NIZO B40 EPS appears to have useful

molecular properties which is illustrated by the desirable rheological properties of the products produced with this strain (Ruas-Madiedo *et al.*, 2002). Moreover, mouse model studies with the EPS from *L. lactis* SBT0495, which has the same structure as NIZO B40 EPS (van Kranenburg *et al.*, 1997), suggested that this EPS reduces cholesterol (Nakajima *et al.*, 1992) and shows anti-tumoral activity (Kitazawa *et al.*, 1991).

Strain NIZO B40 produces phosphorylated EPS with a branched repeating unit containing glucose, galactose and rhamnose. Its EPS producing capacity is encoded on a 42,180-bp EPS-plasmid, pNZ4000, containing the 12-kb *epsRXABCDEFGHIJKL* operon (van Kranenburg *et al.*, 1997, 2000). In addition to these *eps* specific genes, the biosynthesis of the NIZO B40 polymer depends on several household genes that are involved in biosynthesis of the primary EPS precursors, the nucleotide-sugars UDP-glucose, UDP-galactose and dTDP-rhamnose (for recent review see Chapter 1).

In this thesis the modulation of enzymes to engineer EPS production and/or composition were described. To increase EPS production (i) the availability of EPS precursors was stimulated (push strategy) by engineering chromosomally located household genes, and (ii) the expression of the specific EPS production machinery was increased (pull strategy) by engineering the copy number of the plasmid located specific *eps* genes. To study the effect of the push strategy, activities of enzymes were enhanced that are involved at the level of (i) the branching point of sugar breakdown and sugar nucleotide biosynthesis (Chapter 2 and 3), (ii) UDP-glucose and UDP-galactose (Chapter 4), and (iii) dTDP-rhamnose (Chapter 5). The potential of the pull strategy was addressed by increasing the expression of the specific *eps* genes, which led to increased NIZO B40 EPS production (Chapter 6). In this present chapter the potential engineering strategies of sugar metabolism for the production of EPS by LAB are compared and discussed.

Engineering strategies

LAB have already proven to be suitable hosts for metabolic engineering and successful examples are the efficient production of the aroma component diacetyl (Hugenholtz *et al.*, 2000) and the sweetener alanine (Hols *et al.*, 1999). The main advantage of LAB in metabolic engineering strategies relative to many other microbes is based on the nearly complete uncoupling of the basal catabolic and biosynthetic pathways. In contrast to yeast and fungi, the genetics and genetic systems of LAB are relatively simple. There is a limited degree of gene multiplicity. Although, recently three putative but partially inactive paralogs of the lactococcal *ldh* gene were found (Bongers *et al.*, personal communication). This relatively limited gene multiplicity is notably an advantage, when a specific function needs to be disrupted for the desired result. For most of the metabolic engineering successes, the identification of the required modification

was based on 'educated guesses'. This, not strictly rational and partially intuitive approach may be less successful for altering more complex metabolic pathways, such as those involved in EPS production. Therefore, novel tools for the rational design of effective metabolic engineering strategies are required (Stephanopoulos and Kelleher, 2000).

Quantitative approaches like metabolic control analysis (Fell, 1992) and kinetic modeling of metabolic pathways (Theobald *et al.*, 1997) might predict important bottlenecks for production of relevant metabolites. However, a drawback of the modeling approach is that it requires time-consuming mathematical and experimental effort. In this respect, ^{13}C-NMR analysis (Schmidt *et al.*, 1999) is a powerful tool that can generate insight in distribution of different fluxes within a metabolic network. Nevertheless, many metabolic intermediates, relevant enzymes, and kinetic parameters cannot be determined due to insufficient concentrations for proper quantitative analysis or due to a lack of available standards and assays for determining *in vivo* enzyme activities. These are typical drawbacks for modeling of complex pathways like EPS biosynthesis. Therefore, current kinetic modeling possibilities appear to be limited to the central pathways in carbon metabolism such as glycolysis. Recently, some progress towards metabolic control analysis of EPS biosynthesis has been reported (Hoefnagel *et al.*, 2001).

The current explosion of available sequences may accelerate comparative genomics and will subsequently generate predictive metabolic maps of microorganisms. However, this valuable information needs to be followed up by functional analysis to evaluate the functionality of each individual gene. The recent development of genomics based high-throughput technologies, including transcriptomics, proteomics and metabolomics, might allow the efficient analysis of metabolic adaptation upon environmental or genetic changes. In this respect, the use of *in silico* models might be a promising tool for the prediction of effective metabolic engineering of complex metabolism.

Taken together, prediction of metabolic engineering strategies by the use of predictive mathematical or *in silico* models might enhance effective metabolic engineering. However, these modeling tools are still in a developing state of art, and trial and error approaches, notably when they have a high throughput, may provide useful avenues for metabolic engineering. In addition, experimental effort is still required for final validation of every model prediction.

EPS production engineering

LAB polysaccharides are only formed at relatively low production levels compared to currently commercial polymers produced by *Xanthomonas campestris* (Becker *et al.*, 1998). However, the EPS from LAB are very effective biothickeners when produced *in situ* (Hess *et al.*, 1997; Tuinier *et al.*, 2001). To increase EPS production (i) the availability of EPS precursors can be stimulated (push strategy) by engineering chromosomally located household genes and/or (ii)

the efficiency of the EPS production machinery can be improved (pull strategy) by engineering the copy number of the plasmid located specific *eps* genes.

Engineering EPS precursor availability (Push strategy). The intracellular levels of the sugar nucleotides UDP-glucose, UDP-galactose (Chapter 3 and 4) and dTDP-rhamnose (Chapter 5) required for NIZO B40 EPS biosynthesis in *L. lactis* were increased by metabolic engineering. The endogenous activity levels of Pgm, GalU and RfbAC were identified as controlling points in sugar nucleotide production. Overexpression of only *galU* led to eight-fold increase of UDP-glucose and UDP-galactose levels. Similarly, *rfbACBD* overexpression led to two-fold increased dTDP-rhamnose levels. Experiments have been indicated to simultaneously overexpress the *galU* gene and *rfbACBD* genes in the same strain. Therefore, the *galU* gene and the *rfbACBD* genes were cloned under control of the *nisA* promoter (pNZ4125). Changes in the sugar nucleotide levels following induced overexpression of these genes is currently under investigation. The capacity for the lactococcal cell to improve the availability of precursors might be further elevated by introduction of heterologues enzyme analogues with appropriate kinetic parameters (low K_m values) compared to the endogenous ones. Several reports show that the activity level of enzymes involved in sugar nucleotide biosynthesis influences EPS production in *L. lactis* (Gilbert *et al.*, 2000; Levander *et al.*, 2002). In addition, it was found recently that the concentration of UDP-glucose also controls CPS type 3 synthesis in *Streptococcus pneumoniae* (Cartee *et al.*, 2002). Nevertheless, we have not been able to show that increasing intracellular sugar nucleotide pools has a significant effect on the NIZO B40 EPS production level. It is possible that this lack of effect is specific for NIZO B40 EPS and might be different for other lactococcal EPS. Illustrative for this suggestion is the finding of increased *S. pneumoniae* type 3 polysaccharide production levels in *L. lactis* following the increase in GalU enzyme activity level (Gilbert *et al.*, 2000). It would be interesting to analyze the effect of *galU* overexpression in non-rhamnose containing EPS like the galacto-polymer produced by strain NIZO B35 or the glucose and galactose containing polymer produced by strain NIZO B1137 (van Kranenburg *et al.*, 1999a). For convenient selection of the NIZO B35 and B1137 plasmid transformants it is important to mark these plasmids with an erythromycin-resistance gene. In this respect, the *epsL* gene appears to be a good target for integration since this conserved gene has no apparent function in EPS biosynthesis (van Kranenburg *et al.*, 2000). Initial steps were taken to perform this tagging strategy by amplification of internal fragments of the *epsL* genes of the NIZO B35 and NIZO B1137 Eps-plasmids, which were cloned in a pGhost vector (Maguin *et al.*, 1996). Transformants could grow at the non-permissive temperature of the vector, suggesting that marking by a single cross-over event of the pGost derivative into the EPS-plasmid have occurred. Since the replicons of pGhost and pNZ8048 derivatives share the same replicaton, they can not be maintained in the same cell. To circumvent this problem, the erythromycine-resistance gene-cassette can be inserted by a double cross-over event. Alternatively, the NICE system can be inserted into another compatible lactococcal cloning vector, such as pIL253.

Engineering specific *eps* genes (Pull strategy). Previously, it was demonstrated that the overproduction of the NIZO B40 priming glycosyltransferase EpsD in a strain lacking *epsD* in its *eps* gene cluster resulted in an increased EPS production level compared to that of its parent strain (van Kranenburg *et al.*, 1999a). These data suggested that elevation of the level of *eps* gene expression could result in higher EPS production. Indeed, an increase of *eps* gene expression was achieved by elevating the copy number of the Eps-plasmid resulted in almost four-fold elevated NIZO B40 EPS production (Chapter 6). The elevation of Eps-plasmid copy number was achieved by *in trans* overexpression of the *repB* gene and also by cloning of the *eps* gene cluster on the high-copy number vector pIL253 (pNZ4120). Both strategies led to increased *eps* gene expression and subsequently increased EPS production levels, suggesting a correlation between *eps* gene expression and EPS production. Therefore, it is tempting to speculate that the EPS production level could be further improved by increasing the EPS-plasmid copy number even more. For this purpose, the inducible *nisA* gene (De Ruyter *et al.*, 1996), a strong promoter like the *las* promoter (Llanos *et al.*, 1993), or an efficient synthetic promoter (Andersen *et al.*, 2001) could be fused to the *repDEG* operon of pIL253 in analogy to the *repB* overexpression strategy described in Chapter 6. Furthermore, the copy number of lactococcal theta plasmids, such as pNZ4120, is dependent on the host. For pUCL22 derivatives it has been demonstrated that in *L. lactis* strain MG1614, a MG1363 derivative, the copy number is 2-3 per chromosome, while in *L. lactis* strains MMS368 and IL1441 the copy number is 4-6 per chromosome (Frère *et al.*, 1995). Therefore, it would be interesting to evaluate EPS production by using the broad host range vector pNZ4120 in various *L. lactis* strains or even in other LAB or hosts with a low GC-content which harbor all required household genes for NIZO B40 EPS production. This was initiated by transformation of pNZ4120 to *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Bacillus* strains. Although selected strains each of these genera could be transformed by the vector pIL253, no transformants were obtained by transformation of pNZ4120. This is likely to be a technical problem caused by the generally low transformation efficiency with large plasmids (> 10-kb) such as pNZ4120.

Another approach to enhance the expression of the *eps* genes could be performed by fusing one of the above-mentioned promoters to the *eps* gene cluster in pNZ4120 or pNZ4000. Alternatively, *eps* gene expression might be elevated by influencing the level of regulator proteins. The first (*epsR*) and the last (*orfY*) genes of the NIZO B40 *eps* gene cluster encode proteins that are homologous with regulator proteins. However, there is no experimental evidence for any role of these proteins in regulation of *eps* gene expression. Moreover, recent data describe a relationship between tyrosine kinase autophosphorylation and capsule synthesis (Cartee *et al.*, 2002; Morona *et al.*, 2000; Wugeditsch *et al.*, 2000). Phosphorylation state of *S. pneumoniae* CpsD by CpsC regulates capsule synthesis (Cartee *et al.*, 2002; Morona *et al.*, 2000). It is feasible that similar control is mediated in *L. lactis* by EpsB and EpsA, which are the CpsC and CpsD analogues, respectively. Exchange of these analogues might enhance NIZO B40 EPS production. Recently, it was shown that kinetic parameters of glycosyltransferases influence the

binding of capsular polysaccharide precursors (Cartee *et al.*, 2002). It is tempting to speculate that the exchange of glycosyltransferases analogues can improve EPS production level.

Combinatorial push and pull strategies. It would be interesting to combine the push and pull strategies mentioned above to enhance EPS production even more. For this purpose, Eps-plasmid pNZ4120 was transformed to strains overproducing the Pgm, GalU, or GalU and RfbACBD proteins and the NIZO B40 EPS production of the resulting strains grown in presence and absence of the inducer nisin was subsequently analyzed (Table 7.1).

Table 7.1. EPS production of *L. lactis* NZ9000 harboring pNZ4102 (GalU), pNZ4125 (GalU and RfbACBD), pNZ4123 (Pgm), pNZ4030 or pNZ4120 grown in the presence and absence of nisin.

Strain (plasmid)	Eps-plasmid	Nisin (ng ml^{-1})	EPS[a] (mg l^{-1})	(mg $l^{-1} * OD_{600}^{-1}$)
NZ9000	pNZ4030	0	95 ± 3	39 ± 3
NZ9000	pNZ4120	0	303 ± 19	137 ± 7
NZ9000 (pNZ4102)(GalU)	pNZ4120	0	297 ± 10	147 ± 3
		1	320 ± 20	151 ± 9
NZ9000 (pNZ4125)	pNZ4030	0	88 ± 8	36 ± 4
(GalU and RfbACBD)		1	100 ± 4	42 ± 2
	pNZ4120	0	271 ± 8	120 ± 4
		1	295 ± 1	132 ± 2
NZ9000 (pNZ4123)(Pgm)	pNZ4120	0	290 ± 39	128 ± 9
		1	279 ± 6	128 ± 2

[a]) The values are averages based on two independent experiments.

Upon induction with nisin the NIZO B40 EPS production was only slightly increased, indicating that the maximal NIZO B40 EPS production level is determined by the activity level of the factors encoded by the *eps* gene cluster, rather than by the level of sugar nucleotides (Chapter 6). It appears that the level of the EPS production determines the rate of synthesis of the required precursors. One could speculate that the maintenance of the sugar nucleotide levels is demand driven. The observation that the sugar nucleotide levels are lower in EPS-producing cells compared to non EPS-producing cells supports this speculation (Looijesteijn *et al.*, 1999; Ramos *et al.*, 2001). Moreover, the EPS production was decreased in cells with affected growth rates such as in strains carrying inactive *galE* (Chapter 4) or *ccpA* genes (Chapter 3). This indicates that the sugar nucleotide precursors are preferentially used for growth.

EPS structure engineering

To produce polysaccharides with desired properties, one has to know which factors influence these properties and how to modulate them. The knowledge of structure-function relation of polysaccharides in relation to their rheological properties is growing (van Kranenburg *et al.*, 1999b; Tuinier *et al.*, 2001). Important factors that influence the intrinsic viscosity of EPS are molecular mass and chain stiffness (Tuinier *et al.*, 2001). Controlled regulation of the polymerization and export processes or introduction of less flexible sugar linkages might improve the EPS thickening properties.

An approach to influence molar mass is to vary the fermentation conditions. The molar mass of EPS appears to depend on the type of growth limitation during fermentation. Under conditions of carbon limitation the molar mass of EPS produced by strain NIZO B40 and B891 was strongly reduced compared to the EPS produced under conditions of nitrogen or phosphate limitation (Looijesteijn *et al.*, 2000). Current studies focus on the elucidation of the individual role of the genes that are predicted to be involved in export (*epsK*), polymerization (*epsI*) and chain length determination (*epsA*, *epsB*) (M. Nierop Groot, personal communication). These studies might allow directed manipulation of these features and could result in the production of polysaccharides that have shorter or longer chain length compared to the native NIZO B40 polymer. Insight in the mechanism determining the actual size of the polymer might enable the production of EPS with desired molar masses. One could think of oligo-saccharides, which have only limited thickening properties and are subsequently easy to isolate from cultures. These could possibly be used as bodying agents with a high water binding capacity. Furthermore, they can be used as a non-digestible food fraction since it is reported that some EPS are difficult to degrade (Ruijssenaars *et al.*, 2000). In addition, extended polysaccharides are believed to have improved thickening properties (Tuinier *et al.*, 2001). Moreover, lactococcal production of pneumococcal capsular polysaccharides, which are known to be important for virulence, was recently demonstrated by Gilbert *et al.* (2000). It is interestingly to investigate if lactococci can be developed as a vaccine delivery vehicle for mucosal immunisation.

The strong increase in the available *eps* gene cluster sequences from various organisms, including several LAB, has generated tremendous amounts of genetic information, including a large number of glycosyltransferase-encoding genes. This genetic basis, in combination with biochemical information with regard to donor-and acceptor-specificity of individual glycosyl-transferase enzymes, could be exploited. A combinatorial approach of glycosyltransferases for construction of the biosynthetic machinery may result in the production of oligo- and/or polysaccharides in *L. lactis* that have predetermined and novel structure. This approach requires that the export and polymerization machinery do not display a unique specificity for its corresponding native polysaccharide. A promising result in this respect is the observation that the conditional *rfbBD* knock-out resulted in the production of a polysaccharide containing an

altered composition compared to its parental strain (Chapter 5). Similar findings were described for heterologous expression of the *Streptococcus thermophilus* Sfi6 *eps* gene cluster in *L. lactis* which resulted in the production of a polysaccharide containing an altered repeating unit compared the polymer produced in the original host (Stingele *et al.*, 1999). In this respect, the altered EPS obtained from the conditional *rfbBD* mutant was a deliverable of a more targeted approach. These findings indicate that the export and polymerization machinery is not depending on the nature of the polysaccharide. Although various reports have previously established the possibilities to modulate polysaccharide biosynthesis by engineering at the level of specific *eps* genes (for review see van Kranenburg *et al.*, 1999b). The observations that the modulation of the availability of the EPS precursors dTDP-rhamnose (Chapter 5) or UDP-galactoseNAc (Stingele *et al.*, 1999) lead to EPS production with an altered composition, indicate that EPS composition can be influenced by the activities of household enzymes. However, the production levels of altered lactococcal EPS produced to date were low (6-10 mg l^{-1}) in comparison with strains listed in Table 7.1. This suggests that the rate of synthesis of the repeating unit might be reduced by inefficient recognition by the sequential glycosyltransferase machinery. The suggested push and pull strategies above might improve the EPS production level. Finally, future engineering studies including comparative genomics, transcriptomics and metabolomics should clarify the possibilities of these approaches towards the production of tailor made oligo- and polysaccharides.

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Samenvatting

Samenvatting

Van oudsher worden melkzuurbacteriën gebruikt om levensmiddelen langer houdbaar te maken. Doordat deze bacteriën melksuiker omzetten in melkzuur krijgen bijvoorbeeld karnemelk of yoghurt, waarin miljarden van deze bacteriën zich bevinden, hun karakteristieke zure smaak. Naast melkzuur vormen een aantal van deze bacteriën exopolysacchariden (EPS). Dit zijn lange ketens van aan elkaar gekoppelde suikers die gebruikt kunnen worden om de structuureigenschappen van levensmiddelen te verbeteren. Verder zijn er aanwijzingen dat EPS gezondheidsbevorderend zouden zijn. Omdat de meeste melkzuurbacteriën een lange historie van veilig gebruik kennen, is het mogelijk de door deze bacteriën gemaakte EPS als een natuurlijke hulpstof aan producten toe te voegen. *Lactococcus lactis* is een melkzuurbacterie die in de zuivelindustrie veelvuldig wordt gebruikt voor de gewenste omzetting van melksuiker in melkzuur in producten als kaas, kwark, zure room, karnemelk en Scandinavische producten als viili of långfil. Dit proefschrift beschrijft de resultaten van een studie naar de verbetering van de vorming van EPS in de melkzuurbacterie *L. lactis*.

Hoofdstuk 1 beschrijft de huidige kennis omtrent de vorming van EPS, melksuiker en andere suikers. De omzetting van suikers in melkzuur gaat zeer efficiënt in de melkzuurbacterie. Je zou kunnen spreken van een “metabole snelweg”. Omdat slechts een klein deel van alle suikers wordt omgezet in EPS, wordt er in dit hoofdstuk aandacht gegeven aan de omzettingen die de productiviteit van de melkzuurbacterie beperken en die verbeterd zou kunnen worden door gebruik te maken van fysiologische of genetische veranderingen. Speciale aandacht wordt gegeven aan de productie van NIZO B40 EPS. Voor de productie van deze EPS zijn zogenaamde “huishoud-enzymen” nodig voor de aanlevering van de NIZO B40 bouwstenen: UDP-glucose, UDP-galactose en dTDP-rhamnose. Daarnaast zijn specifieke enzymen nodig die deze bouwstenen aan elkaar koppelen tot een karakteristieke repeterende eenheid, polymeriseren en exporteren.

Hoofdstuk 2 beschrijft dat *L. lactis* NIZO B40 ongeveer negen keer meer EPS vormt wanneer glucose in plaats van fructose wordt gebruikt als energiebron in het kweekmedium. Uit verder onderzoek naar enzymactiviteiten bleek dat de lage activiteit van het enzym fructosebifosfatase (Fbp) hiervan de oorzaak was.

Hoofdstuk 3 concentreert zich op het vertakkingspunt van de metabole snelweg richting melksuiker en de omzettingsroute richting EPS. Uit onderzoek naar de enzymen, die rondom dit vertakkingspunt actief zijn, bleek dat het enzym fosfoglucomutase (Pgm) een belangrijke rol speelt in de vorming van de NIZO B40 EPS-bouwstenen UDP-glucose en UDP-galactose. Door de activiteit van Pgm honderd keer te vergroten, verkregen we vier keer zoveel van deze bouwstenen.

Hoofdstuk 4 beschrijft dat twintig keer verhoging van de activiteit van het enzym UDP-glucose pyrofosforylase (GalU) resulteerde in de vorming van acht keer meer van de NIZO B40 EPS bouwstenen UDP-glucose en UDP-galactose. Ook deze activiteitsverhoging had, net zoals bij bovengenoemde verhoging van de Pgm activiteit, geen effect op de vorming van NIZO B40 EPS. Totale uitschakeling van de activiteit van het enzym UDP-galactose epimerase (GalE) leidde tot een veranderd groeigedrag van *L. lactis*. Bovendien bleek in deze mutant de EPS-vorming te stoppen wanneer alleen glucose in het kweekmedium werd aangeboden als suikerbron. Extra toevoeging van galactose gaf weer een normale groei. Bovendien werden er weer EPS gevormd, maar het productieniveau was slechts de helft van die van het normale niveau. Waarschijnlijk komt dit doordat de omzetting van galactose in EPS minder efficiënt is wanneer ook glucose aanwezig is in hetzelfde kweekmedium.

In **Hoofdstuk 5** is aangetoond dat een viertal enzymen (RfbACBD) verantwoordelijk is voor de vorming van dTDP-rhamnose, de derde bouwsteen van NIZO B40 EPS naast UDP-glucose en UDP-galactose. Verhoging van de activiteit van al deze enzymen tegelijkertijd veroorzaakte een verdubbeling van de vorming van deze bouwsteen, maar leidde niet tot een verhoging van de NIZO B40 EPS. Totale uitschakeling van Rfb activiteit overleefde de bacterie niet. Uit verder onderzoek bleek dat als de Rfb activiteit op gecontroleerde wijze werd gereduceerd, de suikersamenstelling van het gevormde EPS anders was dan die van het oorspronkelijke NIZO B40 EPS. Bovendien had het nieuwe EPS andere fysische eigenschappen.

Hoofdstuk 6 concludeert dat de specifieke enzymen een veel grotere rol spelen in de vorming van NIZO B40 EPS dan de huishoud-enzymen die beschreven zijn in voorgaande hoofdstukken. Door de hoeveelheid specifieke enzymen te verhogen, werd de EPS-productie viervoudig verhoogd.

In **Hoofdstuk 7** worden de resultaten van de vorige hoofdstukken samengevat en worden de mogelijkheden belicht die tot praktische toepassingen kunnen leiden.

Nawoord

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Bij het schrijven van het nawoord besef ik dat er nu echt een einde is gekomen aan een enerverende periode. Dit, ongetwijfeld het meest gelezen, onderdeel van het proefschrift is eigenlijk hartstikke leuk om te schrijven, omdat ik om vele herinneringen weer even stil ben (…) of moet lachen (hihi). Natuurlijk doe je onderzoek nooit alleen en er zijn dan ook vele personen die een bijdrage hebben geleverd aan dit boekje. Een aantal van deze personen wil ik in het bijzonder bedanken.

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De NIZO-collega's die met mijn samples gestoeid hebben, zijn: Jan van Riel (HPLC), Roelie Holleman (HPLC), Jan Hoolwerf (Real-time PCR), Fedde Kingma (Slijmerige karnemelk-fermentatie), Marjo Starrenburg (Enzym assays) en Charles Slangen (Vriesdrogen). Jullie werk was een basisvoorziening van het onderzoek, dank voor al jullie inspanningen hiervoor. Verder heb ik pipetteersteun gehad van mijn studenten Martijn van Kaauwen, Tom Holshof, Marit Kosters en mijn Australische collega Barrie Fong Chong. It was a pleasure to work together with you on the ropy strains. Thank you for assisting with the cloning of many constructs.

Veel kennis heb ik opgestoken binnen de verschillende speciale vergadergroepen. Het EPS-project werd binnen NIZO multidisciplinair benaderd. Naast Nel, Remco, Maarten en Saskia wil ik speciaal Ellen Looijesteijn en Marja Kanning noemen. Onze samenwerking blijkt uit meerdere hoofdstukken in dit boekje. Ik vond het erg leuk en stimulerend om met jullie ideeën en technieken uit te wisselen. Ellen, fantastisch dat onze samenwerking ook nog na het EPS-tijdperk voorduurt. De projectgenoten van de WCFS C003-groep wil ik bedanken voor alle suggesties en samenwerking die hieruit voortkwamen. Hierbij heeft Jeroen Hugenholtz een stimulerende rol gespeeld. Marcel, alhoewel we rooskleurige voorspellingen met jouw EPS-model konden maken, hebben we ondervonden dat EPS-biosynthese niet makkelijk te modellen is. De STARLAB bijeenkomsten gingen over de nationale grenzen heen. Een mooie zomer bracht ik door op het lab van Helena Santos in Portugal. Helena and Ana thank you very much for the opportunity to work with NMR in your lab. I will never forget the Figo-goal and my experience with eating a lobster.

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CV

Curriculum Vitae

Ingeborg Catharina Boels werd geboren op 23 november 1970 in Bovensmilde. In 1990 haalde zij haar diploma Atheneum aan de Christelijke Scholengemeenschap voor Assen en omstreken. In datzelfde jaar startte zij aan haar studie Levensmiddelentechnologie, oriëntatie Biotechnologie, aan de toenmalige Landbouwuniversiteit Wageningen. Tijdens de doctoraalfase verrichtte zij onderzoek bij de vakgroepen Levensmiddelenchemie (prof. dr. ir. A. G. J. Voragen; dr. ir. M. Mutter) en Industriële Microbiologie (prof. dr. ir. J. A. M. de Bont; dr. G. J. Grobben). Daarnaast deed zij een stage aan de Lund University in Zweden (prof. dr. B. Hahn-Hägerdal; dr. N. Q. Meinander) en een stage bij Unilever Research in Vlaardingen (dr. M. J. A. de Groot). Eind 1996 werd het ingenieursdiploma behaald en begin 1997 begon zij bij NIZO *food research* aan het promotie-onderzoek dat in dit proefschrift beschreven staat (prof. dr. W. M. de Vos; dr. M. Kleerebezem). Vanaf juni 2002 is zij werkzaam bij Friesland Coberco Dairy Foods te Deventer.

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Addendum

Addendum

The research described in this thesis was carried out at NIZO *food research* in Ede, and was part of the research carried out in the scientific programme “Processing & Functionality” and “Microbial Functionality & Safety” of the Wageningen Centre for Food Sciences. Furthermore, the research was part of the Graduate School VLAG (Food Technology, Nutrition & Health Sciences). Part of this work was supported by the E. C. research Grant BIOT-CT96-0498.

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