Physiology of exopolysaccharide biosynthesis by *Lactococcus lactis*

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Abstract

Several lactic acid bacteria (LAB) produce exopolysaccharides (EPS). EPSs produced by LAB are a potential source of natural additives and because LAB are food grade organisms, these EPSs can also be produced *in situ*. The amount of EPS in milk fermented with strain NIZO B40, which produces an anionic EPS composed of glucose, rhamnose, galactose and phosphate, is very low. Optimising the culture conditions and medium composition could increase this relatively low concentration. Using pH-controlled fermentations and a chemically defined medium, the total EPS production was highest at pH 5.8 and 25°C. Glucose was demonstrated to be the most efficient sugar source for EPS production by *L. lactis* NIZO B40. With fructose as the sugar source only a minor amount of EPS was produced. The intracellular levels of sugar nucleotides, the EPS precursors, were much lower in fructose- than in glucose-grown cultures. The activity of the enzymes involved in the biosynthesis of the sugar nucleotides were however unaffected by the source of sugar but the activity of fructose-1,6-bisphosphatase (FBPase) was very low. FBPase catalyses the conversion of fructose-1,6-diphosphate into fructose-6-phosphate, an essential step for the biosynthesis of sugar nucleotides from fructose but not from glucose. Overexpression of the *fbp* gene resulted in increased EPS synthesis on fructose.

Most culture conditions influenced growth as well as EPS formation and EPS synthesis itself was also influenced by the growth rate. EPS production by strain NIZO B40 starts at the exponential growth phase but continues during the stationary phase in batch cultures, indicating that EPS biosynthesis and growth are not strictly coupled. Indeed we found that non-growing cultures were still able to produce EPS, making it possible to study the influence of different culture conditions on EPS biosynthesis independent of growth.

The amounts of EPS produced by *L. lactis* NIZO B40 and NIZO B891 were comparable under glucose and leucine limitation. The efficiency of EPS production, that is the quantity of EPS produced per quantity of glucose consumed was, however, much higher under conditions of glucose limitation. The production of phosphorylated B40 EPS and of unphosphorylated B891 EPS was strongly reduced under conditions of phosphate limitation. The sugar composition of both B40 and B891 EPS and the phosphate content of B40 EPS were unaffected by the type of limitation but, glucose limitation resulted in the production of EPSs with strongly reduced molecular masses.

Anionic B40 EPS in suspension and a cell-associated layer of this EPS protected the bacteria against toxic copper ions and nisin, probably due to charge interactions. Furthermore, cell-associated EPS resulted in a decrease in the sensitivity of the bacteria to bacteriophages and lysozyme, most likely by masking the targets for the phages and the enzyme. The protection of EPS against nisin and bacteriophages could be a competitive advantage in mixed strain dairy starter cultures. Unfortunately, the EPS yields were not increased in the presence of copper, bacteriophages, nisin or lysozyme.

Aan mijn ouders

Contents

Chapter 1

General Introduction

Polysaccharides and oligosaccharides are important components of food products. They act for instance as gel- and thickening agents, stabilisers and non-digestible fibres. Many of the polysaccharides in food products are derived from plants and seaweeds but, bacterial exopolysaccharides (EPS) are also an important source of additives that are used for manipulation of the texture of these products. EPSs produced by lactic acid bacteria (LAB) have a good potential as thickening agents and it is even suggested that they have beneficial effects on human health (Sikkema and Oba, 1998). LAB include the genera *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus*. These organisms are Gram-positive, non-sporing microaerophilic bacteria that use pyruvate as an electron acceptor, resulting in the production of lactate (Malik *et al.*, 1993). LAB play an essential role in a large variety of food fermentations. Many different strains are used as starter cultures for dairy, meat, vegetable and bakery products resulting in increased shelf life of these products and the formation of flavour compounds. In most fermented dairy products, the activity of the LAB also has a large impact on the texture of the products, synthesis of EPS being one of these activities. LAB have a long tradition in food application and are generally regarded as food grade organisms. This allows not only the use of EPSs produced by these organisms as natural additives but also *in situ* production of the thickening agents.

The ability to produce EPS is widely spread in nature. The first part of this introduction deals with bacterial EPSs in general: their chemical composition, physiological function and biosynthesis as well as the similarities in the formation of EPSs with the production of other bacterial polysaccharides. This part will include EPSs like xanthan and gellan, which are already applied successfully as natural additives. The second part deals with EPS production by LAB. The elucidated structures of EPSs produced by LAB are presented, the genetics and biosynthesis of these EPSs are discussed as well as the physical properties of the EPSs and the culture conditions that influence their production.

Bacterial exopolysaccharides

Bacteria produce a number of polysaccharides.

a) Storage polysaccharides that are located in the cytoplasm such as glycogen.
b) Structural polysaccharides like peptidoglycan and (lipo)teichoic acid.
c) Lipopolysaccharides (LPS) that are cell surface polysaccharides characteristic for Gram-negative bacteria. LPSs have a carbohydrate chain linked to a lipid anchor embedded in the bacterial membrane.
d) EPSs

Bacterial EPSs can either be excreted in the extracellular environment as slime with little or no cell association or form an adherent cohesive layer that is often covalently bound. In the latter case, the polysaccharides are also called capsular polysaccharides (CPS; Whitfield and Valvano, 1993).

EPSs are either homopolysaccharides consisting of only one type of sugar or heteropolysaccharides composed of several chemically different monosaccharides. Due to variations in the monosaccharide composition, the presence of different non-carbohydrate substituents, the linkage types and the degrees of polymerisation and branching, a very diverse range of chemical structures of EPSs and hence physical properties is possible. Monosaccharide(derivatives)s that are found in bacterial EPSs include amongst others D-glucose, D-galactose, D-mannose, L-rhamnose, L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid and L-guluronic acid (Shibaev, 1986; Sutherland, 1994). The sugar moieties in the EPSs can be substituted with both organic and inorganic molecules. Organic components that often occur in EPSs are acyl substituents, most commonly acetate and pyruvate (Sutherland 1994). Phosphate and to a lesser extent sulphate are possible inorganic substituents in EPSs.

Physiological role of bacterial exopolysaccharides

The physiological function of EPS has not been established clearly. EPSs are mostly not essential for the micro-organisms that produce them because enzymatic or physical removal of EPS generally does not negatively affect cell growth *in vitro* and in addition mutants unable to produce EPS occur spontaneously (Schellhaass, 1983). EPSs do not serve as reserve sources of energy and carbon since EPS-producing bacteria are mostly not capable of degrading their own EPSs but unrelated organisms sometimes catabolise polysaccharides of other bacteria (Dudman, 1977).

Proposed functions of EPSs are mostly of a protective nature. EPSs can bind a high volume of water and provide bacteria with a hydrophilic physical barrier. This layer could slow down dehydration processes and in that way protect the bacteria against desiccation (Roberson and Firestone, 1992; Ophir and Gutnick, 1994). An EPS layer could also be a protective barrier against the penetration of antibiotics (Costerton *et al.*, 1987; Allison and Matthews, 1992) and of toxic metal ions (Dudman, 1977) but EPS could also mediate in the uptake of useful metal ions (Weiner *et al.*, 1995).

CPSs are potent virulence factors by protecting the bacteria from phagocytosis and decreasing their sensitivity for humoral and cellular host defence mechanisms (Costerton *et al.*, 1987; Moxon and Kroll, 1990). It has also been suggested that EPSs may play a role in protection of the bacteria against bacteriophage attack (Cerning, 1990; Forde *et al.*, 1999) but on the other hand, bacteriophages exist which use EPS as a receptor. The latter phages can generally only adhere on EPS-producing bacteria and not on their non-EPS-producing mutants (Lindberg, 1977; Cerning, 1990).

EPSs are also involved in bacteria-plant interactions. Capsular polysaccharides produced by several phytopathogenic bacteria are essential for their virulence (Roberts, 1996) and the symbiotic relationship between nitrogen-fixing bacteria, especially *Rhizobium meliloti*, and their host plants greatly depends on EPSs (Roberts, 1996; Mendrygal and González, 2000).
EPSs might increase the adhesion of bacteria to solid surfaces and to each other which facilitates the formation of biofilms (Boyd and Chakrabarty, 1995; Roberts, 1996; Cammarota and Sant'Anna, 1998). These biofilm or adhesion functions of EPSs could result in many industrial and medical problems like plugging of filters, corrosion of metals, fouling of pipes, the initiation of dental caries and attachment of pathogenic bacteria to their target cells (Costerton *et al.*, 1987).

It is obvious that one type of EPS can not fulfil all the functions that are described above. The exact role of an EPS is probably dependent on the natural environment of the micro-organisms. The biosynthesis of EPS could be a response to selective pressures in these natural environments and provide a competitive advantage (Whitfield, 1988). EPS produced by soil bacteria are more likely involved in the protection against desiccation, whereas the role of EPS synthesised by marine bacteria probably lies in sequestering of essential nutrients from the dilute nutrient environment (Dudman, 1977).

Biosynthesis of bacterial exopolysaccharides

EPSs are produced via two distinct pathways that differ in the location of the synthesis and the nature of the precursors:

1) Homopolysaccharides that are produced outside the bacterial cell

The biosynthesis of most homopolysaccharides takes place outside the bacterial cell surface by enzymes that are either secreted by the bacteria or are loosely associated with the cell surface (Cerning, 1995). Bacteria that produce these polysaccharides utilise a specific substrate, mostly sucrose, for the biosynthesis of polyglucose and polyfructose. The energy needed for polymerisation comes from the hydrolysis of sucrose (de Vuyst and Degeest, 1999). Although the producing organisms could grow on other substrates, these cannot be used to produce the polymers (Sutherland, 1982). The best known example of an EPS synthesised via this pathway is dextran, an α-glucan produced by amongst others *Leuconostoc mesenteroides*. Other examples include mutan, a polyglucose produced by *Streptococcus* species and levan, a polyfructose produced by for instance *Streptococcus salivarius* and *Lactobacillus reuteri* LB 121 (Cerning, 1990; Van Geel-Schutten *et al.*, 1999).

2) EPSs composed of repeating units

The biosynthesis of EPSs composed of repeating units starts with the intracellular formation of building blocks, the sugar nucleotides. These activated sugars, mostly

nucleotide diphosphate monosaccharides, serve as activated glycosyl donors (Shibaev, 1986). Most enzymes needed for the biosynthesis of the sugar nucleotides are not unique for EPS biosynthesis but are housekeeping enzymes that are also involved in the synthesis of cell wall polysaccharides. These enzymes are either found in the cytoplasm or are loosely associated with the cytoplasmic membrane (Whitfield, 1988; Whitfield and Valvano, 1993). Two different mechanisms have been described for the polymerisation of activated sugars: a block mechanism and a monomeric mechanism (Shibaev, 1986; Whitfield and Valvano, 1993; Whitfield and Roberts, 1999). The mechanisms differ in the cellular location of their polymerisation steps and in the direction of chain polymerisation.

Block mechanism

When the block mechanism is used for the formation of a polysaccharide, sugar precursors are not directly transferred to a growing polymer chain but oligosaccharide intermediates are formed. The sequential transfer of sugar-1-phosphate, followed by sugars from the sugar nucleotides to a lipid carrier, undecaprenyl monophosphate, by the sequential activities of glycosyltransferase enzymes assembles the oligosaccharide repeating units (Troy, 1979; Sutherland, 1982; Whitfield and Valvano, 1993; Whitfield and Roberts, 1999). The repeating units vary in size from di- to heptasaccharides and could also contain non-sugar molecules (Cerning, 1995). The oligosaccharide repeating units are transported through the cytoplasmic membrane by a flippase or Wzx protein (Whitfield and Roberts, 1999). Polymerisation of the repeating units occurs at the reducing end of a growing chain at the outer face of the cytoplasmic membrane and is catalysed by the enzyme Wzy in *E. coli* (Whitfield and Roberts, 1999). One polyprenyl diphosphate molecule is liberated after polymerisation of two repeating units and can be used as an acceptor again after dephosphorylation (Roberts, 1996). Examples of EPSs produced in this way are xanthan produced by *Xanthomonas campestris* and succinoglycan produced by *Rhizobium meliloti* (Harding, 1993; Shibaev, 1986).

Monomeric mechanism

For the monomeric mechanism, sugar monomers are sequentially transferred from the corresponding glycosyl donors to a growing polysaccharide that could be attached to undecaprenyl. In this mechanism, the polymeric chain grows from the non-reducing end (Shibaev, 1986; Whitfield and Valvano, 1993; Whitfield and Roberts, 1999). The polymerisation occurs at the inner face of the cytoplasmic membrane. ATP-binding cassette (ABC) transporters translocate the complete nascent polymers across this membrane (Roberts, 1996; Whitfield and Roberts, 1999). Hyaluronic-acid capsular polysaccharides of streptococci and group II capsular polysaccharides of *E. coli* are produced in this way (Whitfield, 1995; Roberts, 1996).

Post-polymerisation

After polymerisation, using either of the two mechanisms mentioned above, monosaccharide residues may be enzymatically modified or side chains may be added to

an originally linear polymer. The latter is only possible when the formation of the main chain is independent of the presence of side chains. When the incorporation of monosaccharide residues present in side chains is necessary for elongation of the chain, the intermediate formation of a linear polysaccharide does not occur (Shibaev, 1986; Whitfield and Valvano, 1993). The final stages of the biosynthetic process may include transfer of a polysaccharide chain from an intermediate acceptor to a final acceptor for CPS, or liberation of the free polysaccharide. Polysaccharides produced by Gram-negative bacteria need to be translocated across the outer membrane (Whitfield and Valvano, 1993).

Other systems

There are exceptions to the general biosynthetic pathways for bacterial EPSs as described above. Alginate is a linear polymer composed of mannuronic acid and guluronic acid residues. In algae, GDP-guluronic acid serves as sugar donor for the guluronic acid residues in this EPS. However, in *Azotobacter vinelandii* the production of alginate differs because it is originally synthesised as polymannuronic acid. Outside the bacterial cell, an epimerase converts some of the mannuronic acid residues to guluronic acid. This is the one known exception in micro-organisms where a residue of a particular monosaccharide in EPS does not require the formation of a nucleotide precursor of that monosaccharide (Whitfield and Valvano, 1993).

Examples of commercialised exopolysaccharides

Several EPSs produced by fermentation, such as dextran, xanthan, and gellan, are commercially used for a variety of food and non-food applications (Fett *et al.*, 1996).

Dextran

Dextran, an α-glucan, has become the first industrially-produced polysaccharide of microbial origin and has many non-food applications. This polysaccharide was discovered because its presence resulted in thickening and gelation of sugar cane and beet syrups. The organism responsible for dextran production in the syrups was identified as *Leuconostoc mesenteroides* in 1878 (Crescenzi, 1995).

Xanthan

For successful use of an EPS as a thickening agent in food, low concentrations of the polysaccharide should provide a high viscosity but it is also often desired that the products flow after shaking, pouring or stirring. Furthermore, the EPSs need to be resistant to wide ranges of temperature, pH and ionic strength and should be compatible with other food ingredients.

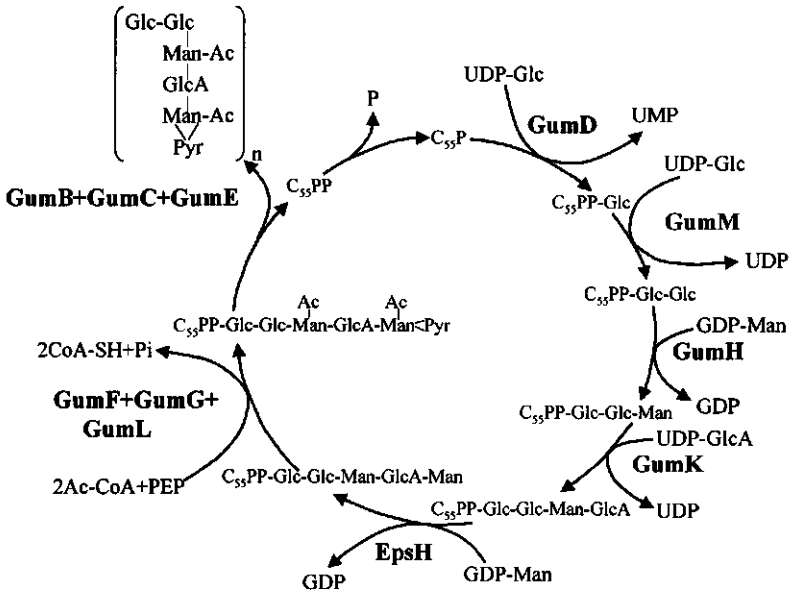


Figure 1.1

Proposed pathway for the biosynthesis of xantham gum. C_{55}-P, isoprenoid lipid carrier; Glc, glucose; GlcA, glucuronic acid; Man, mannose; Ac, acetyl; Pyr, pyruvyl; (Becker *et al.*, 1998).

Xanthan, produced by the plant-pathogenic bacterium *Xanthomonas campestris*, meets all these requirements. The use of xanthan as a food additive was approved in 1969 (Morris, 1993). Xanthan is widely used as a thickener or viscosifier in both food and non-food products and is also used as a stabiliser for a wide variety of suspensions, emulsions and foams (Becker *et al.*, 1998). Xanthan gum is a relatively inexpensive product because of the high conversion of substrate to polymer (Sutherland, 1998). The EPS is an acidic, high molecular mass (0.9-1.6 * 10^6 Da) heteropolysaccharide with a repeating unit consisting of a poly-β1,4-glucose backbone with trisaccharide side chains on alternate glucose residues. The side chains, composed of mannose and glucuronic acid residues, are substituted with pyruvate and acetate. The degree of substitution depends on the bacterial strain and on the culture conditions (Sutherland, 1994). Biosynthesis of the repeating units of xanthan requires the intracellular formation of the sugar nucleotides UDP-glucose, GDP-mannose and UDP-glucuronic acid. Acetyl-CoA and phosphoenolpyruvate serve as precursors for the acetate and pyruvate groups, which are added at the level of the lipid-linked pentasaccharides (Fig. 1.1; Harding *et al.*, 1993; Becker *et al.*, 1998). The xanthan molecules adopt an ordered double-helical conformation in solution that is responsible for many of the rheological characteristics of the polymer (Sutherland, 1998). The most important industrial process for recovery of

xanthan from the fermentation liquid involves pasteurisation to destroy the bacterial cells and enzymes followed by precipitation of xanthan with alcohol. Subsequently the xanthan precipitate is spray-dried and milled to a powder (Becker *et al.*, 1998).

Gellan

Another industrially EPS which is accepted as a food additive is gellan, a broad spectrum gelling agent produced by *Sphingomonas paucimobilis* strains (Sutherland, 1998). The linear gellan molecules are build up from tetrasaccharide repeating units composed of glucose, glucuronic acid and rhamnose in a ratio of 2:1:1 for which UDP-glucose, UDP-glucuronic acid and dTDP-rhamnose serve as precursors, respectively (Morris, 1993; Martins and Sá-Correia, 1993). The polymer of about 500 kD carries O-acetyl and glyceryl substituents. Deacetylation of native gellan causes extensive intermolecular association resulting in enhanced gel stiffness in solution. Deacetylated gellans form rigid brittle, thermoreversible gels at low polymer concentrations in the presence of mono- or divalent cations (Sutherland, 1998).

Shared pathways and control of exopolysaccharide biosynthesis

Lipopolysaccharides (LPS) produced by Gram-negative bacteria are composed of an O-antigen that is anchored to lipid A in the outer membrane via an oligosaccharide core (Wright and Kanegasaki, 1971). The synthesis of the O-antigen of LPS as well as the biosynthesis of cell wall polysaccharides such as peptidoglycan and teichoic acid, proceed via identical pathways as described for the biosynthesis of EPSs composed of repeating units (Shibaev, 1986). Due to the similarity in the production of cell wall polysaccharides and EPS, both processes depend on the uptake of substrate, energy supply and the availability of sugar nucleotides and undecaprenyl phosphate.

Control of EPS production is very complex and is possible at different levels. Although it is realised that the different factors also influence each other, factors influencing EPS production can roughly be divided in: availability of intermediates, enzyme activities and gene expression.

Availability

A bacterial cell contains a limited amount of undecaprenyl phosphate. The availability of these molecules may be a controlling factor of the biosynthesis of EPSs which are formed through the mediation of isoprenoid lipid intermediates (Sutherland, 1982). In most cells there is presumed to be sufficient isoprenoid lipid present to permit the simultaneous synthesis of all different polysaccharides. In bacteria where EPS is produced at the end of the exponential growth phase or during the stationary phase, it is possible that there is insufficient isoprenoid lipid to allow simultaneous production of polysaccharides necessary for growth and EPS during the exponential growth phase. The production of EPS is frequently enhanced under

suboptimal growth conditions and this too could be a result of increased lipid availability, because under these conditions less undecaprenyl phosphate is required for the biosynthesis of cell wall polymers (Sutherland, 1982).

Enzyme activities

Control of EPS production could also be possible by regulation of the enzymes involved in its production. These enzymes are involved either in the initial carbohydrate metabolism, or are enzymes involved in sugar nucleotide synthesis and interconversion, glycosyltransferases, translocases and polymerases (Sutherland, 1972). The group of enzymes that is involved in the synthesis of activated sugar has been shown to control exopolysaccharide synthesis in several organisms like *E. coli*, *Sphingomonas paucimobilis*, *Pseudomonas aeruginosa* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Grant *et al.*, 1970; 1993; Ashtaputre and Shah, 1995; Leitão and Sá-Correia, 1995; Grobben *et al.*, 1996). These enzymes were, however, not involved in regulation of EPS production by *Klebsiella aerogenes* and *Pseudomonas* NCIB 11264 (Norval and Sutherland, 1973; Williams and Wimpenny, 1980).

Gene expression

Control of EPS synthesis at the genetic level has also been shown for many, mainly Gram-negative organisms. Genes for the biosynthesis of cell-surface polysaccharides are usually arranged in clusters of one or more transcriptional units. The biosynthetic gene clusters encode glycosyltransferases, proteins involved in polymerisation and export as well as enzymes needed for the formation of unique sugar nucleotides. Genes involved in the regulation of EPS synthesis are mostly not encoded within this cluster (Withfield and Valvano, 1993; Roberts, 1996).

Exopolysaccharides produced by lactic acid bacteria

Many LAB are capable of producing EPS. Homopolysaccharides, like dextran, mutan and levan, produced by several species of *Lactobacillus*, *Streptococcus* and *Leuconostoc* have already been mentioned before. Hetero- or homopolysaccharides composed of repeating units are produced by a large group of LAB. EPS-producing LAB have technological significance in the production of several fermented dairy products.

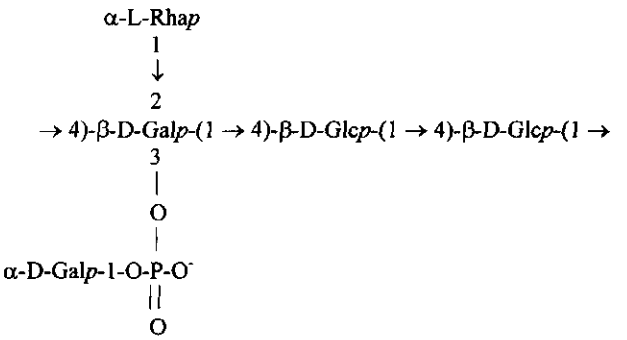
In yoghurt, exopolymer-producing cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* contribute to a smooth texture, improve the viscosity, decrease the susceptibility to syneresis and prevent gel fracture (Schellhaass, 1983). This is especially important in countries like Holland and France where addition of plant or animal stabilisers to unfruited yoghurt is prohibited (Cerning *et al.*, 1986). Mucoid lactococci have traditionally been used in starter cultures for Scandinavian fermented milk products such as, långfil, viili, and taette. The characteristic ropiness of these products is a result of the presence of EPS. The loss of the desired viscosity due to the spontaneous loss of the EPS-

producing ability of the starter strains is a reoccurring problem during the manufacturing of the ropy sour milks. The instability of the EPS-producing trait in lactococci is a consequence of the involvement of plasmid encoded genes (Malik *et al.*, 1993). Polysaccharide-producing strains of *Lactobacillus* are used in the manufacture of kefir. In kefir grains, EPS is believed to be responsible for embedding of bacterial and yeast cells (Yokoi *et al.*, 1990). This is necessary because the grains are recovered, dried and re-used for many successive inoculations (Schellhaass, 1983).

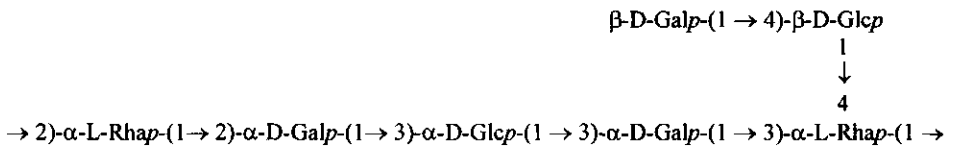
The amounts of EPS produced by LAB in milk vary widely and depend on the species as well as on the strains (Cerning, 1995). Generally the conversion of substrate into EPS composed of repeating units by LAB is relatively low compared to other EPS producers like *Xanthomonas campestris*. The structures of EPSs produced by LAB are quite diverse. Fig. 1.2 depicts the primary structures of EPSs produced by LAB that have been elucidated so far.

Lactococcus

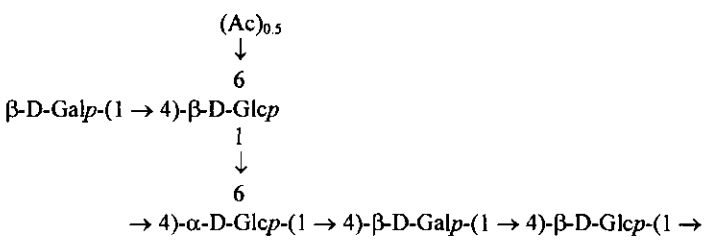
1) *Lactococcus lactis* subsp. *cremoris* NIZO B40 (van Casteren *et al.*, 1998) and SBT0495 (Nakjima *et al.*, 1992):



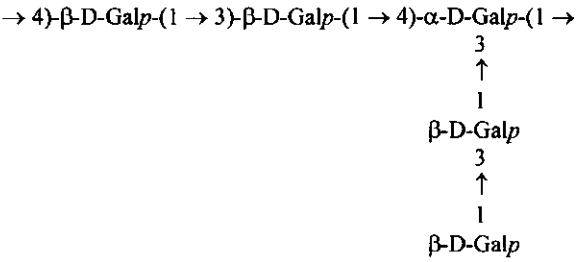
2) *Lactococcus lactis* subsp. *cremoris* NIZO B39 (van Casteren *et al.*, 2000a):



3) *Lactococcus lactis* subsp. *cremoris* NIZO B891 (van Casteren *et al.*, 2000b):

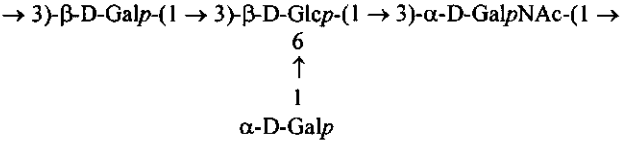


4) *Lactococcus lactis* subsp. *cremoris* H414 (Gruter *et al.*, 1992):

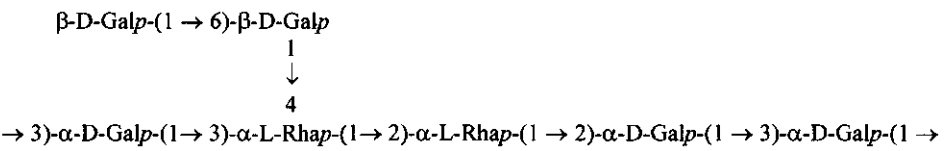


Streptococcus

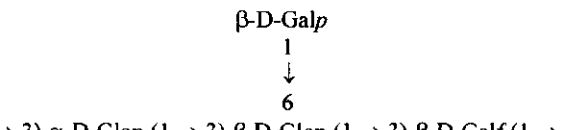
1) *Streptococcus thermophilus* Sfi6 (Doco *et al.*, 1990; Stingele *et al.*, 1996):



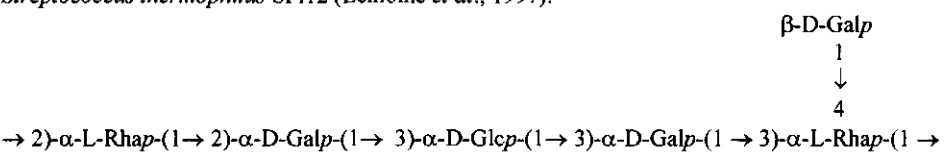
2) *Streptococcus thermophilus* OR901, Rs and Sts (Faber *et al.*, 1998):



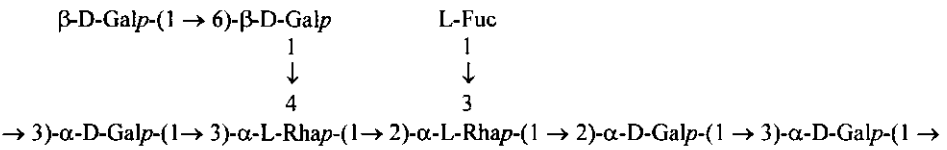
3) *Streptococcus thermophilus* SFi39 (Lemoine *et al.*, 1997):



4) *Streptococcus thermophilus* SFi12 (Lemoine *et al.*, 1997):

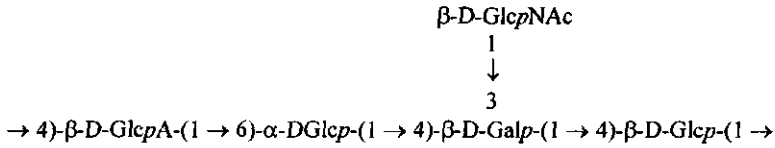


5) *Streptococcus thermophilus* MR-1C (Low *et al.*, 1998):

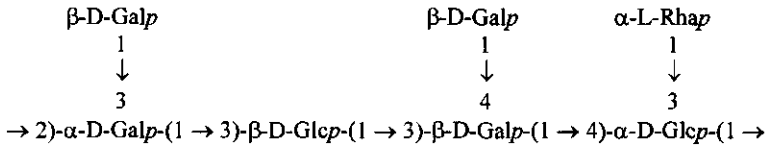


Lactobacillus

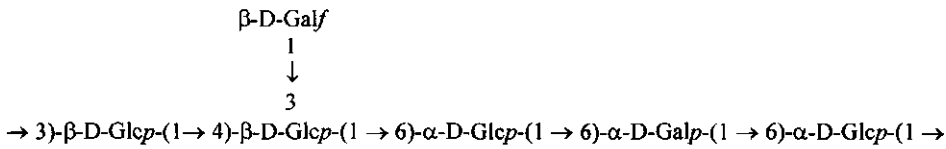
1) *Lactobacillus acidophilus* LMG9433 (Robijn *et al.*, 1996b):



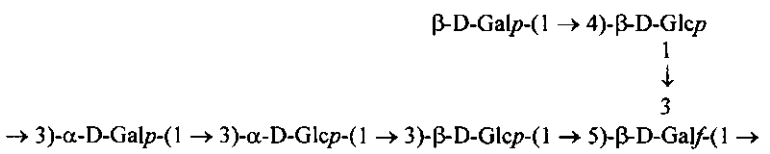
2) *Lactobacillus delbrueckii* subsp. *bulgaricus* rr (Gruter *et al.*, 1993):



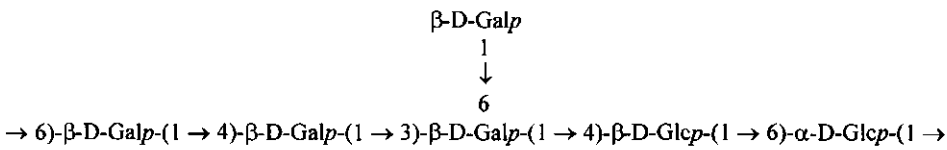
3) *Lactobacillus helveticus* 766 (Robijn *et al.*, 1995b):



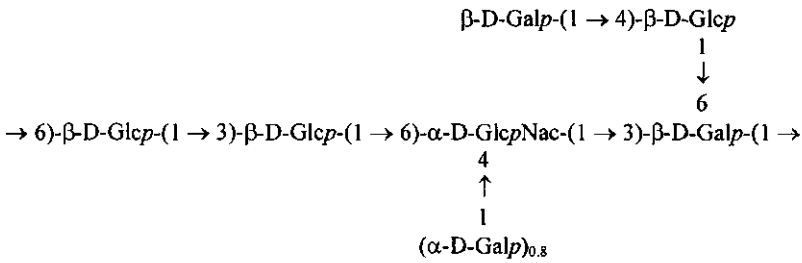
4) *Lactobacillus helveticus* TN-4 (Yamamoto *et al.*, 1995) and Lh59 (Stingele *et al.*, 1997):



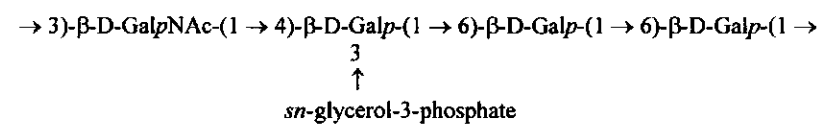
5) *Lactobacillus helveticus* ssp. (Staaf *et al.*, 1996):



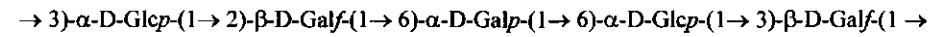
6) *Lactobacillus helveticus* TY1-2 (Yamamoto *et al.*, 1995):



7) *Lactobacillus paracasei* 34-1 (Robijn *et al.*, 1996a):



8) *Lactobacillus rhamnosus* C83 (Vanhaverbeke *et al.*, 1998):



9) *Lactobacillus sake* 0-1 (Robijn *et al.*, 1995a):

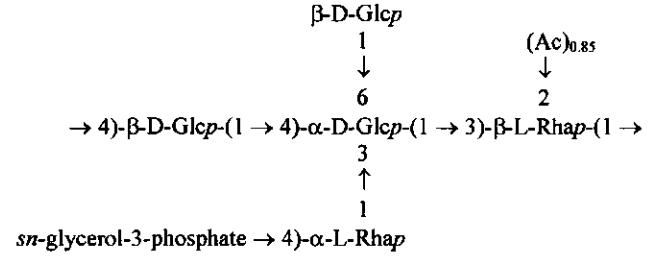


Figure 1.2

Primary structures of the repeating units of EPSs produced by LAB. Glc, glucose; Gal, galactose; Rha, rhamnose; GlcNAc, N-acetyl-glucosamine; GalNAc, N-acetyl-galactosamine; GlcA, glucuronic acid; Fuc, fucose; Ac, acetyl. The configuration (D and L) and pyranose (*p*) and furanose (*f*) structure are indicated.

Influence of culture conditions and medium composition on exopolysaccharide production by lactic acid bacteria

Exopolysaccharides are generally produced under all culture conditions but the amounts and sometimes even the structures of the EPSs that are produced, are influenced by the growth conditions and the medium composition. In the paragraph 'shared pathways and control of EPS production' it was mentioned that the production of cell wall polysaccharides and EPS production proceed via identical pathways and that regulation of EPS production is complex. From the examples mentioned in this paragraph it can be concluded that there is no single set of culture conditions that results in optimal EPS yields for all EPS-producing LAB. The amount of EPS that is produced by a certain strain depends on i.e. the incubation temperature, the pH and the carbon source:

Incubation temperature

The incubation temperature often profoundly influences the biosynthesis of EPS. Sutherland (1972) postulated that slower growth as a result of a reduced incubation

temperature, results in increased EPS synthesis due to an increased availability of lipid intermediates. Indeed, incubation of mesophilic LAB at 25°C instead of 30°C yielded 50 to 60% more EPS (Cerning *et al.*, 1992). Increased EPS production at reduced incubation temperatures was also observed for *Lactobacillus rhamnosus* C83 (Gamar *et al.*, 1997), *Lactobacillus sake* 0-1 (van den Berg *et al.*, 1995) and for one of the EPSs produced by *Lactococcus lactis* subsp. *cremoris* LC330 (Marshall *et al.*, 1995). Temperatures above as well as temperatures below the optimal growth temperature resulted in enhanced EPS production by *Streptococcus salivarius* subsp. *thermophilus* (Gancel and Novel, 1994). However, in another lactic acid bacterium, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, it was demonstrated that the specific EPS production increased with increasing temperature (Grobben *et al.*, 1995; Garcia-Garibay and Marshall, 1991). The rate of EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* RR was lower at 30°C than at 40°C although the maximal concentration of EPS was similar for both temperatures (Kimmel and Roberts, 1998).

pH

The pH also influences the EPS production in various LAB. In pH-controlled fermentations, the specific EPS production by *Lactobacillus casei* CRL 87 was highest at pH 4.0 (Mozzi *et al.*, 1996) and optimal pHs of 5.8 and 6.2 were found for EPS production by, respectively, *Lactobacillus sake* 0-1 (van den Berg *et al.*, 1995) and *Lactobacillus rhamnosus* (Gamar-Nourani *et al.*, 1998). On the other hand, EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 was not affected by culture pH (Grobben *et al.*, 1995).

Carbon source

The EPS yield of LAB depends on the carbon source as well. Of all the carbon sources tested, *Lactobacillus rhamnosus* C83 used mannose or a combination of glucose and fructose most efficiently for the production of EPS (Gamar *et al.*, 1997), glucose was by far the most effecient carbon source for EPS production by *Lactobacillus casei* CG11 (Cerning *et al.*, 1994) and galactose resulted in the highest EPS production by *Lactobacillus casei* CRL 87 (Mozzi *et al.*, 1995) The specific EPS production was inversely correlated with the total growth of *Streptococcus salivarius* subsp. *thermophilus*, more EPS was produced on glucose or fructose, on which the strain grew poorly, than on lactose or sucrose, which resulted in fast growth (Gancel and Novel, 1994). The opposite was true for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, where fructose and mannose utilising cells showed a strongly reduced specific EPS production as well as biomass formation compared with the preferred sugars glucose and lactose (Grobben *et al.*, 1995). Generally the structures of EPSs produced by LAB are unaffected by the source of sugar. However, Cerning *et al.* (1994) and Grobben *et al.* (1996) showed that the sugar composition of EPS produced by *Lactobacillus casei* CG11

and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, respectively, are influenced by the source of sugar. For the latter organism, the difference in the monomeric sugar composition of the EPS produced on glucose and fructose was the result of the absence on fructose of one of the two EPSs that were produced on glucose (Grobben *et al.*, 1997).

Other medium components

The presence or absence of other medium components may also influence EPS biosynthesis. Mozzi *et al.* (1995) reported that the presence of $MnSO_4$ and/or $CaCl_2$ stimulated EPS production by *Lactobacillus casei* CRL 87. For *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 it was demonstrated that omission of certain vitamins reduced total growth but increased the specific EPS production (Grobben *et al.*, 1998).

EPS production phase

The growth phase in which EPS is produced is also strain-dependent. EPS production by *Lactobacillus rhamnosus* C83 (Gamar *et al.*, 1997), *Lactobacillus delbrueckii* subsp. *bulgaricus* RR (Kimmel and Roberts, 1998) and *Streptococcus thermophilus* LY03 (de Vuyst *et al.*, 1998) was growth related. *Lactobacillus sake* 0-1 produced EPS only during the exponential phase (Van den Berg *et al.*, 1995) and *Streptococcus salivarius* subsp. *thermophilus* only during the stationary phase (Gancel and Novel, 1994). EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* strains CNRZ 1187 and NCFB 2772, *Lactobacillus casei* CG11 and *Lactococcus lactis* subsp. *cremoris* LC330 started during exponential phase and continued there after (Bouzar *et al.*, 1996; Grobben *et al.*, 1998; Kojic *et al*, 1992; Marshall *et al.*, 1995).

Biosynthesis and genetics of exopolysaccharide composed of repeating units by lactic acid bacteria

Biosynthesis

LAB produce EPSs with a wide variety of chemical structures (Fig. 1.2). Monomers that are found in these EPSs are glucose, galactose, rhamnose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid and fucose as well as phosphate and acetate. The biosynthesis of the EPSs starts with the intracellular formation of EPS building blocks, the sugar nucleotides, from sugar-1-phosphate. Sugar nucleotides are also essential for the biosynthesis of cell wall polymers and hence many of the enzymes involved in their production are not unique to EPS formation. Some of these enzymes have been reported to influence the rate of EPS production as well as the composition of EPS produced by LAB. In *Lactococcus lactis* a relationship between UDP-galactose 4-epimerase activity and mucoidity was found as well as an inverse relationship between UDP-glucose pyrophosphorylase activity and mucoidity (Forsén and Häivä, 1981). EPS levels were, however, not quantified in

this study. Culturing of *Lactobacillus delbrueckii* subsp. *bulgaricus* in medium containing either glucose or fructose resulted in the production of EPSs with different sugar compositions. The lack of rhamnose in EPS produced by fructose-grown cultures was the result of the absence of activity of enzymes involved in the synthesis of dTDP-rhamnose (Grobben *et al.*, 1996).

Glycosyl transferases catalyse the formation of repeating units by sequential addition of the sugar residues from the sugar nucleotides to isoprenoid glycosyl lipid carriers which are embedded in the cytoplasmic membrane. The lipid intermediate involved in the biosynthesis of viilian by *Lactococcus lactis* subps. *cremoris* SBT 0495 was identified as undecaprenol (Oba et al., 1999a). A model for the formation of lipid linked repeating units produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 is given as an example in Fig.1.3. EPS produced by this organism is similar to that produced by strain SBT 0495 and the genes encoding EPS production in both strains are the same (van Kranenburg et al., 1999b). However, using a different method Oda et al. (1999a) found a different order for the biosynthesis of the repeating units by strain SBT 0495. The repeating units are translocated across the cytoplasmic membrane and polymerised. Finally, the polymer chains are liberated from the cells.

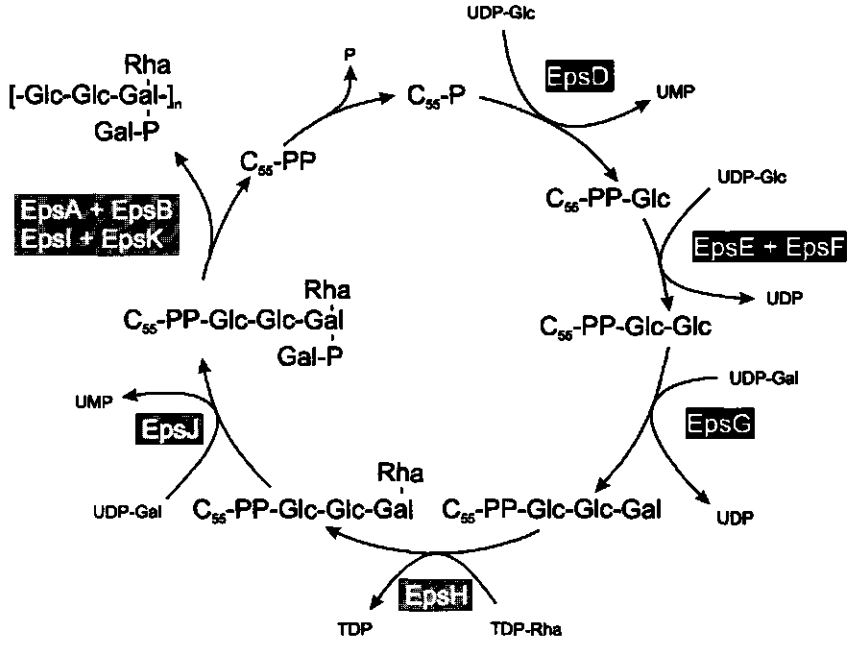


Figure 1.3

Model for EPS biosynthesis in *Lactococcus lactis* subsp. *cremoris* NIZO B40. The functionality of the genes in the black boxes has been proven the functions of the other genes are predicted based on their homology with genes involved in the biosynthesis of polysaccharides in other bacteria. C_{55}-P, lipid carrier; Glc, glucose; Gal, galactose; Rha, rhamnose. (van Kranenburg, 1999).

Genetics

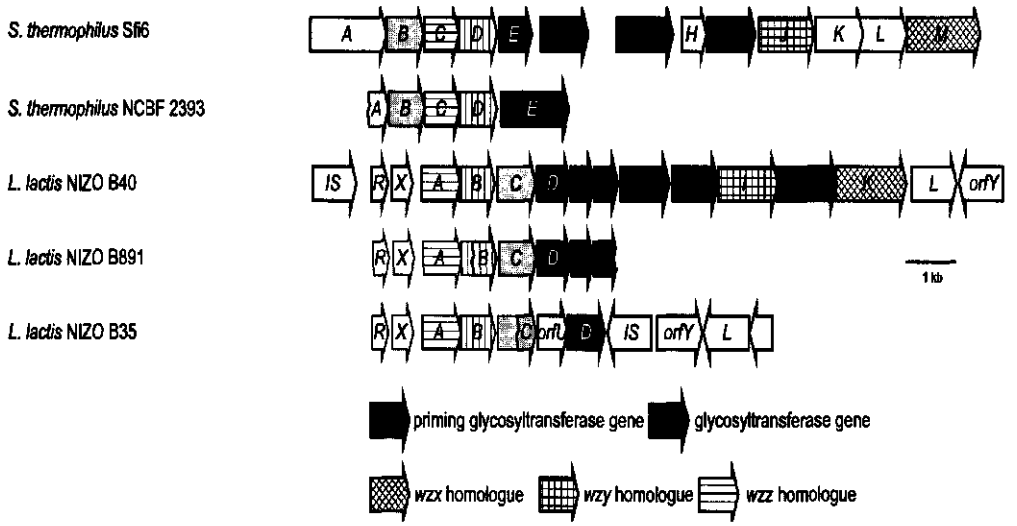


Figure 1.4

Genetic organisation of the *eps* gene clusters of *Streptococcus thermophilus* Sfi6, NCFB 2393 and *Lactococcus lactis* subsp. *cremoris* NIZO B40, B891 and B35. Glycosyltransferase genes and *wzx*, *wzy* and *wzz* homologues are indicated. Wzx, Wzy and Wzz are predicted to be involved in export of the repeating units through the cytoplasmic membrane, polymerisation of the repeating units and chain length determination, respectively. Adapted from van Kranenburg *et al.* (1999c).

The genetics of EPS production by dairy LAB has extensively been studied for *Streptococcus thermophilus* (Stingele *et al.*, 1996; 1999) and *Lactococcus lactis* (van Kranenburg *et al.*, 1997; 1999ab). EPS biosynthesis by *Streptococcus thermophilus* Sfi6 involves 13 genes, *eps A* to *M*, that are sequentially arranged on a 14 kb fragment of the Sfi6 chromosome (Figure 1.4). The genes show homology with genes responsible for regulation, chain-length determination, biosynthesis of the repeating unit, polymerisation and export of other polysaccharide-producing bacteria (Stingele *et al.*, 1996). EpsE, a phosphogalactosyl-transferase, catalyses the first step in the biosynthesis of the EPS repeating unit of *Streptococcus thermophilus* Sfi6 and EpsG is responsible for the following step by linking the second sugar, N-acetylgalactosamine (Stingele *et al.*, 1999). EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 is encoded by a 12 kb gene cluster on a 42 kb plasmid, pNZ4000. This *eps* gene cluster contains 14 genes with the order *epsRXABCDEFGHIJKL* (Fig. 1.4; van Kranenburg *et al.*, 1997; 1999b). The gene product EpsD is a glucosyl transferase, responsible for linking the first sugar of the repeating unit to the lipid acceptor. Secondly, EpsE and EpsF link glucose to the lipid-linked glucose. Linking of galactose from UDP-galactose to the lipid-linked glucose molecules by EpsG completes the assembly of the trisaccharide backbone of the repeating units (van Kranenburg, 1999; Fig. 1.3). The functions

of the other genes encoded within the *eps* gene cluster are predicted based on their homology with genes involved in the biosynthesis of polysaccharides by other bacteria (van Kranenburg *et al.*, 1999a). The genetic organisation of EPS production by other LAB resembles that of strain NIZO B40 (Fig. 1.4; van Kranenburg *et al.*, 1999c).

Functional properties of exopolysaccharides produced by lactic acid bacteria

Studies on the physical properties of EPS produced by LAB are rather scarce. However, these properties are very important for the efficiency of EPSs as thickening or gelling ingredients. In this perspective, some EPSs produced by LAB are promising. For instance the EPS produced by *Lactobacillus sake* 0-1 (Fig. 1.2) has, at a concentration of 1%, a higher apparent viscosity than a solution of 1% xanthan gum over a range of shear rates from 0 to 300 s^{-1} (van den Berg *et al.*, 1995). The commercially available xanthan that was used in this study had a molar mass between 4-9 x 10^6 Da and the average molar mass of the *Lactobacillus sake* 0-1 EPS was determined to be 6 x 10^6 Da. The shear-thinning properties of the pure *Lactobacillus sake* 0-1 EPS were comparable with that of xanthan. The hydrodynamic volume and thus intrinsic viscosity of the negatively charged 0-1 EPS is influenced by repulsion effects of the intramolecular negative charges. Addition of NaCl shielded the charged groups and resulted in a decrease of the repulsion effects, a smaller hydrodynamic volume and hence a lower intrinsic viscosity (van den Berg *et al.*, 1995).

The viscoelastic properties of the native and partially purified viilian, produced by *Lactococcus lactis* subsp. *cremoris* SBT 0495 (Fig. 1.2), were studied using dynamic and steady shear measurements. Viilian has an approximate molar mass of 2 x 10^6 Da. The results indicated that viilian can be regarded as a random coil polysaccharide because aqueous solutions of this polysaccharide behaved as an intermediate between a liquid system and a weak gel and is shear thinning. The dependence of the viscosity on the shear rate found for this EPS was in agreement with that found for guar and xanthan. Viilian did not show a temperature-dependent conformational change in the temperature range of 5-70°C (Oba *et al.*, 1999b).

The physical properties of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 were studied in detail by Tuinier *et al.* (1999abf; 2000). All the studies were performed in aqueous solutions at an ionic strength of 0.1 M, which resembles the ionic strength of many food products. The primary structure of B40 EPS is identical to villian (Fig. 1.2). The number-averaged molar mass and radius of gyration of the B40 EPS were found to be 1.5 x 10^6 g mol^{-1} and 86 nm, respectively (Tuinier *et al.*, 1999a). Furthermore, it was shown that the viscosity and shear thinning behaviour of B40 EPS and other random coil polysaccharides can be predicted by the EPS concentration, molar mass and hydrodynamic radius of the polymer (Tuinier *et al.*, 1999b). The influence of EPSs on the rheological properties of products depends on the characteristics of the EPS itself (molar mass and size) as well as on the interactions of the EPS with the other components of the product. For that reason the

interactions of EPS with the most relevant colloidal particles present in dairy products (casein micelles, whey proteins and fat globules) were also studied (Tuinier *et al.*, 1999cdef).

The three studies that are mentioned above show that the rheological properties of EPSs produced by LAB are quite promising but, the conversion rates of substrate into EPS need to be increased before the EPSs could be economically used as food additives. Nevertheless, the different EPSs are effective thickeners since relatively low concentrations of the EPSs resulted in a high viscosity. The low EPS production levels might be sufficient for the use of EPS-producing LAB for the *in situ* production of biothickeners. EPS that is produced *in situ* by yoghurt bacteria positively influenced the texture of yoghurt (Hess *et al.*, 1997). However, the presence of EPS alone does not guarantee ropiness: the molar mass of the EPSs and the growth characteristics of the yoghurt cultures also play an important role in determination of the ropiness of yoghurt (Faber *et al.*, 1998; van Marle, 1998).

Outline of this thesis

The aim of this study was to optimise the production of EPS by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and to obtain insight in the biosynthetic pathway and the regulation of EPS production. *Lactococcus lactis* was chosen as the producing organism because amongst LAB, the metabolism and genetics of *Lactococcus lactis* have been studied most extensively. *Lactococcus lactis* strains are the most important organisms in starter cultures for the production of Dutch cheeses. The organoleptic properties of the cheese depend largely upon the metabolism of the lactococci. Furthermore, the homofermentative *Lactococcus lactis* strains are used for the production of fermented milk, quark, sour cream, butter and buttermilk. Many metabolic properties relevant to industry, such as EPS production, are plasmid encoded in this organism. Strain NIZO B40 was selected for this study as it produced EPS of a known sugar composition and the genetics of EPS production were partly characterised (van Kranenburg, 1999). Simultaneous with the work presented in this thesis, the physical properties (Tuinier, 1999), chemical structure (van Casteren, 2000) and biodegradability (Ruijssenaars *et al.*, 2000) of EPSs produced by *Lactococcus lactis* subsp. *cremoris* have been investigated.

In this introduction, it was shown that the influence of culture conditions and medium composition on EPS production depends on the producing organism. Chapter 2 describes the determination of the optimal conditions for EPS production by strain NIZO B40. The influence of the carbon source, incubation temperature, culture pH, growth rate and several stress conditions on the EPS yield and EPS composition were studied in a chemically defined medium using pH-controlled fermentations. It was shown that EPS production by strain NIZO B40 continued during the stationary phase and was not strictly coupled to growth. This property allowed the study of the influence of different culture conditions on EPS production independent of growth as is also described in this chapter.

It is often assumed that limitation of nutrient sources other than the carbon source will result in increased EPS synthesis. In order to further optimise the EPS production, the influence of glucose, nitrogen and phosphate limitation on EPS production as well as EPS composition was studied using chemostat cultures as is described in chapter 3. The molecular mass of an EPS is very important for its rheological properties. It was observed that the molecular mass of lactococcal EPS was affected by applying different limitations.

Regulation of EPS production by the source of sugar is the subject of chapter 4. *Lactococcus lactis* NIZO B40 produced much more EPS on glucose than on fructose as sugar substrate. Biosynthesis of polysaccharides produced by lactococci starts with the intracellular formation of EPS-precursors, the sugar nucleotides, followed by the formation of the repeating units and transport and polymerisation of the repeating units. Regulation of EPS production could be possible at all of the steps involved in its biosynthesis. We studied the regulation of EPS production by the source of sugar by determining the activities of enzymes involved in the biosynthesis of the sugar nucleotides as well as the activity of the *eps* genes, the intracellular levels of the sugar nucleotides on both substrates and the activities of enzymes involved in the initial sugar metabolism. It was demonstrated that the initial sugar metabolism was responsible for the difference in EPS production by glucose- and fructose-grown cultures.

The physiological function of lactococcal EPS is the subject of chapter 5. In order to determine this role, the tolerance of a non-EPS-producing *Lactococcus lactis* strain and an EPS-producing isogenic variant of this strain to several stress factors such as increased temperatures, freezing, freeze-drying, antibiotics, bacteriophages, metal ions, lysozyme and digestion was compared. The aim of this work was to obtain insight in the physiological function of EPS in order to find conditions that could be used to improve the EPS yields.

Finally, the results of the work described in the chapters 2 to 5 are discussed in chapter 6.

REFERENCES

Allison, D.G. and Matthews, M.J. 1992. Effect of polysaccharide interactions on antibiotic susceptibility of *Pseudomonas aeruginosa*. J. Appl. Bacteriol. **73:** 484-488.

Ashtaputre, A.A. and A.K. Shah. 1995. Studies on the exopolysaccharide from *Shingomonas paucimobilis*-GS1: Nutritional requirement and precursor-forming enzymes. Curr. Microbiol. **31:** 234-238.

Becker, A., Katzen, F., Pühler, A. and Ielpi, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl. Microbiol. Biotechnol. **50:** 145-152.

Bouzar, F., Cerning, J. and Desmazeaud, M. 1996. Exopolysaccharide production in milk by *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 1187 and by two colonial variants. J. Dairy Sci. **79:** 205-211.

Boyd, A. and Chakrabarty, A.M. 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Ind. Microbiol. **15:** 162-168.

Cammarota, M.C. and Sant'Anna Jr., G.L. 1998. Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol. Lett. **20:** 1-4.

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87:** 113-130.

Cerning, J. 1995. Production of exopolysaccharides by lactic acid bacteria and dairy propionibacteria. Lait **75:** 463-472.

Cerning, J., Bouillanne, C. and Desmazeaud, M.J. 1986. Isolation and characterization of exocellular polysaccharide produced by *Lactobacillus bulgaricus*. Biotechnol. Lett. **8:** 625-628.

Cerning, J., Bouillanne, C., Landon, M. and Desmazeaud, M. 1992. Isolation and characterization of exopolysaccharides from slime-forming mesophilic lactic acid bacteria. J. Dairy Sci. **75:** 692-699.

Cerning, J., Renard, C.M.G.C., Thibault, J.F., Bouillanne, C., Landon, M., Desmazeaud, M. and Topisirovic, L. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. Appl. Environ. Microbiol. **60:** 3914-3919.

Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. **41:** 435-464.

Crescenzi, V. 1995. Microbial polysaccharides of applied interest: Ongoing research activities in Europe. Biotechnol. Prog. **11:** 251-259.

de Vuyst, L., Vanderveken, F., van de Ven, S. and Degeest, B. 1998. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. J. Appl. Microbiol. **84:** 1059-1068.

de Vuyst, L. and Degeest, B. 1999. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev. **23:** 153-177.

Doco, T., Wieruszeski, J., Fournet, B., Carcano, D., Ramos, P. and Loones, A. 1990. Structure of an exocellular polysaccharide produced by *Streptococcus thermophilus*. Carbohydr. Res. **198:** 313-321.

Dudman, W.F. 1977. The role of surface polysaccharides in natural environments. In: *Surface carbohydrates of the prokaryotic cell*, ed. Sutherland, I.W., pp. 357-414, Academic Press, London.

Faber, E.J., Zoon, P., Kamerling, J.P. and Vliegenthart, J.F.G. 1998. The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures. Carbohydr. Res. **310:** 269-276.

Fett, W.F., Osman, S.F., Fishman, M.L. and Ayyad, K. 1996. Biopolymers from fermentation. In: *Agricultural materials as renewable resources: nonfood and industrial applications*, ACS Symposium Series No. 647, ed. Fuller, E.G., McKeon, T.A. and Bills, D.D., pp. 76-87, Washington.

Forde, A., Daly, C. and Fitzgerald, G.F. 1999. Identification of four phage resistance plasmids from *Lactococcus lactis* subsp. *cremoris* HO2. Appl. Environ. Microbiol. **65:** 1540-1547.

Forsén, R. and Häivä, V.-M. 1981. Induction of stable slime-forming and mucoid states by *p*-fluorophenylalanine in lactic streptococci. FEMS Microbiol. Lett. **12:** 409-413.

Gamar, L., Blondeau, K. and Simonet, J.-M. 1997. Physiological approach to extracellular polysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. **83:** 281-287.

Gamar-Nourani, L., Blondeau, K. and Simonet, J.-M. 1998. Influence of culture conditions on exopolysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. **85:** 664-672.

Gancel, F. and Novel, G. 1994. Exopolysaccharide production by *Streptococcus salivarius* ssp. *thermophilus* cultures. 1. Conditions of production. J. Dairy Sci. 77: 685-688.

Garcia-Garibay, M. and Marshall, V.M.E. 1991. Polymer production by *Lactobacillus delbrueckii* ssp. *bulgaricus*. J. Appl. Bacteriol. **70:** 325-328.

Grant, W.D., Sutherland, I.W. and Wilkinson, J.F. 1970. Control of colanic acid synthesis. J. Bacteriol. **103:** 89-96.

Grobben, G.J., Sikkema, J., Smith, M.R. and de Bont, J.A.M. 1995. Production of extracellular polysaccharides by *Lactobacillus delbrueckii* ssp. bulgaricus NCFB 2772 grown in a chemically defined medium. J. Appl. Bacteriol. **79:** 103-107.

Grobben, G.J., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1996. Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. Appl. Microbiol. Biotechnol. **46:** 279-284.

Grobben, G.J., van Casteren, W.H.M., Schols, H.A., Oosterveld, A., Sala, G., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1997. Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Appl. Microbiol. Biotechnol. **48:** 516-521.

Grobben, G.J., Chin-Joe, I., Kitzen, V.A., Boels, I.C., Boer, F., Sikkema, J., Smith, M.R. and de Bont, J.A.M. 1998. Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 with a simplified defined medium. Appl. Environ. Microbiol. **64:** 1333-1337.

Gruter, M., Leeflang, B.R., Kuiper, J., Kamerling, J.P. and Vliegenthart, F.G. 1992. Structure of the exopolysaccharide produced by *Lactococcus lactis* subspecies *cremoris* H414 grown in a defined medium or skimmed milk. Carbohydr. Res. **231:** 273-291.

Gruter, M., Leeflang, B.R., Kuiper, J., Kamerling, J.P. and Vliegenthart, J.F.G. 1993. Structural characterisation of the exopolysaccharide produced by *Lactobacillus delbrückii* subspecies *bulgariucs* rr grown in skimmed milk. Carbohydr. Res. **239:** 209-226.

Harding, N.E., Raffo, S., Raimondi, A., Cleary, J.M. and Ielpi, I. 1993. Identification, genetic and biochemical analysis of genes involved in synthesis of sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. **139:** 447-457.

Hess, S.J., Roberts, R.F. and Ziegler, G.R. 1997. Rheological properties of nonfat yogurt stabilized using *Lactobacillus delbrueckii* ssp. *bulgaricus* producing exopolysaccharide or using commercial stabilizer systems. J. Dairy Sci. **80:** 252-263.

Kimmel, S.A. and Roberts, R.F. 1998. Development of a growth medium suitable for exopolysaccharide production by *Lactobacillus delbrueckii* ssp. *bulgaricus* RR. Int. J. Food Microbiol. **40:** 87-92.

Kojic, M., Vujcic, M., Banina, A., Cocconcelli, P., Cerning, J. and Topisirovic, L. 1992. Analysis of exopolysaccharide production by *Lactobacillus casei* CG11, isolated from cheese. Appl. Environ. Microbiol. **58:** 4086-4088.

Leitão, J.H. and Sá-Correia, I. 1995. Growth-phase-dependent alginate synthesis, activity of biosynthetic enzymes and transcription of alginate genes in *Pseudomonas aeruginosa*. Arch. Microbiol. **163:** 217-222.

Lemoine, J., Chirat, F., Wieruszeski, J.M., Strecker, G., Favre, N. and Neeser, J.R. 1997. Structural characterization of the exocellular polysaccharides produced *by Streptococcus thermophilus* SFi39 and SFi12. Appl. Environ. Microbiol. **63:** 3512-3518.

Lindberg, A.A. 1977. Bacterial surface carbohydrates and bacteriophage adsorption. In: *Surface carbohydrates of the prokaryotic cell*, ed. Sutherland, I.W., pp. 289-356, Academic Press, London.

Low, D., Ahlgren, J.A., Horne, D., McMahon, D.J., Oberg, C.J. and Broadbent, J.R. 1998. Role of *Streptococcus thermophilus* MR-1C capsular exopolysaccharide in cheese moisture retention. Appl. Environ. Microbiol. **64:** 2147-2151.

Malik, R.K., Prasher, R. and Mathur, D.K. 1993. Genetics and production of extracellular polysaccharides by lactic acid bacteria – a review. Indian J. Dairy Sci. **47:** 987-994.

Marshall, V.M., Cowie, E.N. and Moreton, R.S. 1995. Analysis and production of two exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* LC330. J. Dairy Res. **62:** 621-628.

Martins, L.O. and Sá-Correia, I. 1993. Temperature profiles of gellan gum synthesis and activities of biosynthetic enzymes. Biotechnol. Appl. Biochem. **20:** 385-395.

Mendrygal, K.E. and González, J.E. 2000. Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. J. Bacteriol. **182:** 599-606.

Morris, V.J. 1993. Fermentation-derived polysaccharides for use in foods. J. Chem. Technol. Biotechnol. **58:** 199-201.

Mozzi, F., Savoy de Giori, G., Oliver, G. and Font de Valdez, G. 1995. Exopolysaccharide production by *Lactobacillus casei*. II. Influence of the carbon source. Milchwissenschaft **50:** 307-309.

Mozzi, F., de Giori, G.S., Oliver, G. and de Valdez, G.F. 1996. Exopolysaccharide production by *Lactobacillus casei* under controlled pH. Biotechnol. Lett. **18:** 435-439.

Moxon, E.R. and Kroll, J.S. 1990. The role of bacterial polysaccharide capsules as virulence factors. Curr. Top. Microbiol. Immunol. **150:** 65- 85.

Nakajima, H., Hirota, T., Toba, T., Itoh, T. and Adachi, S. 1992. Structure of the extracellular polysaccharide from slime forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. Carbohydr. Res. **224:** 245-253.

Norval. M. and I.W. Sutherland. 1973. The production of enzymes involved in exopolysaccharide synthesis in *Klebsiella aerogenes* types 1 and 8. Eur. J. Biochem. **35:** 209-215.

Oba, T., Doesburg, K.K., Iwasaki, T. and Sikkema, J. 1999a. Identification of biosynthetic intermediates of the extracellular polysaccharide viilian in *Lactococcus lactis* subspecies *cremoris* SBT 0495. Arch. Microbiol. **171:** 343-349.

Oba, T., Higashimura, M., Iwasaki, T., Matser, A.M., Steeneken, P.A.M., Robijn, G.W. and Sikkema, J. 1999b. Viscoelastic properties of aqueous solutions of the phosphopolysaccharide "viilian" from *Lactococcus lactis* subsp. *cremoris* SBT 0495. Carbohydr. Polym. **39:** 275-281.

Ophir, T. and Gutnick, D.L. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl. Environ. Microbiol. **60:** 740-745.

Roberson, E.B. and Firestone, M.K. 1992. Relationship between desiccation and exopolysaccharide production in soil *Pseudomonas* sp. Appl. Environ. Microbiol. **58:** 1284-1291.

Roberts, I.S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Ann. Rev. Microbiol. **50:** 285-315.

Robijn, G.W., van den Berg, D.J.C., Haas, H., Kamerling, J.P. and Vliegenthart, J.F.G. 1995a. Determination of the structure of the exopolysaccharide produced by *Lactobacillus sake* 0-1. Carbohydr. Res. **276:** 117-136.

Robijn, G.W., Thomas, J.R., Haas, H., van den Berg, D.J.C., Kamerling, J.P. and Vliegenthart, J.F.G. 1995b. The structure of exopolysaccharide produced by *Lactobacillus helveticus* 766. Carbohydr. Res. **276:** 137-154.

Robijn, G.W., Wienk, H.L.J., van den Berg, D.J.C., Haas, H., Kamerling, J.P. and Vliegenthart, J.F.G. 1996a. Structural studies of the exopolysaccharide produced by *Lactobacillus paracasei* 34-1. Carbohydr. Res. **285:** 129-139.

Robijn, G.W., Gallego, R.G., van den Berg, D.J.C., Haas, H., Kamerling, J.P. and Vliegenthart, J.F.G. 1996b. Structural characterization of the exopolysaccharide produced by *Lactobacillus acidophilus* LMG9433. Carbohydr. Res. **288:** 203-218.

Ruijssenaars, H.J., Stingele, F. and Hartmans, S. 2000. Biodegradability of food-associated extracellular polysaccharides. Curr. Microbiol. **40:** 194-199.

Schellhaass, S.M. 1983. Characterization of exocellular slime produced by bacterial starter cultures used in the manufacture of fermented dairy products. Ph.D. Thesis, University of Minnesota, U.S.A.

Shibaev, V.N. 1986. Biosynthesis of bacterial polysaccharide chains composed of repeating units. Adv. Carbohydr. Chem. Biochem. **44:** 277-339.

Sikkema, J. and Oba, T. 1998. Extracellular polysaccharides of lactic acid bacteria. Snow Brand R&D Rep. **107:** 1-31.

Staaf, M., Widmalm, G., Yang, Z. and Huttunen, E. 1996. Structural elucidation of an extracellular polysaccharide produced by *Lactobacillus helveticus*. Carbohydr. Res. **291:** 155-164.

Stingele, F. Neeser, J.-R. and Mollet, B. 1996. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. J. Bacteriol. **178:** 1680-1690.

Stingele, F., Lemoine, J. and Neeser, J.-R. 1997. *Lactobacillus helveticus* Lh59 secretes an exopolysaccharide that is identical to the one produced by *Lactobacillus helveticus* TN-4, a presumed spontaneous mutant of *Lactobacillus helveticus* TY1-2. Carbohydr. Res. **302:** 197-202.

Stingele, F., Vincent, S.J.F., Faber, E.J., Newell, J.W., Kamerling, J.P. and Neeser, J.-R. 1999. Introduction of the exopolysaccharide gene cluster from *Streptococcus thermophilus* Sfi6 into *Lactococcus lactis* MG1363: production and characterization of an altered polysaccharide. Mol. Microbiol. **32:** 1287-1295.

Sutherland, I.W. 1972. Bacterial exopolysaccharides. Adv. Microb. Physiol. **8:** 143-213.

Sutherland, I.W. 1982. Biosynthesis of microbial exopolysaccharides. Adv.Microb. Physiol. **23:** 79-150.

Sutherland, I.W. 1994. Structure-function relationship in microbial exopolysaccharides. Biotech. Adv. **12:** 393-448.

Sutherland, I.W. 1998. Novel and established applications of microbial polysaccharides. Tibtech **16:** 1-46.

Troy II, F.A. 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Ann. Rev. Microbiol. **33:** 519-560.

Tuinier, R. 1999. An exocellular polysaccharide and its interactions with proteins. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Tuinier, R., Zoon, P., Olieman, C., Cohen Stuart, M.A., Fleer, G.J. and de Kruif, C.G. 1999a. Isolation and physical characterization of an exocellular polysaccharide. Biopolymers **49:** 1-9.

Tuinier, R., Zoon, P., Cohen Stuart, M.A., Fleer, G.J. and de Kruif, C.G. 1999b. Concentration and shear-rate dependence of an exocellular polysaccharide. Biopolymers **50:** 641-646.

Tuinier, R., ten Grotenhuis, E., Holt, C., Timmins, P.A. and de Kruif, C.G. 1999c. Depletion interaction of casein micelles and an exocellular polysaccharide. Phys. Rev. E **60:** 848-856.

Tuinier, R. and de Kruif, C.G. 1999d. Phase behavior of casein micelles/exocellular polysaccharide mixtures: experiment and theory. J. Chem. Phys. **110:** 9996-9304.

Tuinier, R. and de Kruif, C.G. 1999e. Phase separation, creaming, and network formation of oil-in-water emulsions induced by an exocellular polysaccharide. J. Colloid Interface Sci. **218:** 201-210.

Tuinier, R., Oomen, C.J., Zoon, P., Cohen Stuart, M.A. and de Kruif, D.G. 2000. Viscoelastic properties of an exocellular polysaccharide. Biomacromolecules, in press.

Van Casteren, W.H.M. 2000. Structural characterisation and enzymic modification of exopolysaccharides from Lactococcus lactis. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

van Casteren, W.H.M., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1998. Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40. Carbohydr. Polym. **37:** 123-130.

van Casteren, W.H.M., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 2000a. Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39. Carbohydr. Res. **324:** 170-181.

van Casteren, W.H.M., de Waard, P., Dijkema, C., Schols, H.A. and Voragen A.G.J. 2000b. Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B891. Carbohydr. Res. **327:** 55-66.

van den Berg, D.J.C., Robijn, G.W., Janssen, A.C., Giuseppin, M.L.F., Vreeker, R., Kamerling, J.P., Vliegenthart, J.F.G., Ledeboer, A.M. and Verrips, C.T. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. Appl. Environ. Microbiol. **61:** 2840-2844.

van Geel-Schutten, G.H., Faber, E.J., Smit, E., Bonting, K., Smith, M.R., ten Brink, B., Kamerling, J.P., Vliegenthart, J.F.G. and Dijkhuizen, L. 1999. Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl. Environ. Microbiol. **65:**3008-3014.

Vanhaverbeke, C., Bosso, C., Colin-Morel, P., Gey, C., Gamar-Nourani, L., Blondeau, K., Simonet, J.M. and Heyraud, A. 1998. Structure of an extracellular polysaccharide produced by *Lactobacillus rhamnosus* strain C83. Carbohydr. Res. **314:** 211-220.

van Kranenburg, R. 1999. Exopolysaccharide biosynthesis in *Lactococcus lactis*; a molecular characterisation. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

van Kranenburg, R., Marugg, J.D., van Swam, I.I., Willem, N.J. and de Vos, W.M. 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactocccus lactis*. Mol. Microbiol. **24:** 387-397.

van Kranenburg, R., van Swam, I.I., Marugg, J.D., Kleerebezem, M. and de Vos, W.M. 1999a. Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: Functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. J. Bacteriol. **181:** 338-340.

van Kranenburg, R., Vos, H.R., van Swam, I.I., Kleerebezem, M. and de Vos, W.M. 1999b. Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other gram-positive cocci: complementation, expression, and diversity. J. Bacteriol. **181:** 6347-6353.

van Kranenburg, R., Boels, I.C., Kleerebezem, M. and de Vos, W.M. 1999c. Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. Curr. Opin.Biotechnol. **10:** 498-504.

van Marle, M. 1998. Structure and rheological properties of yoghurt gels and stirred yoghurts. Ph.D. Thesis, University of Twente, Enschede, The Netherlands.

Weiner, R., Langille, S. and Quintero, E. 1995. Structure, function and immunochemistry of bacterial exopolysaccharides. J. Ind. Microbiol. **15:** 339-346.

Whitfield, C. 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. **34:** 415-420.

Whitfield, C. 1995. Biosynthesis of lipopolysaccharide O-antigens. Trends Microbiol. **3:** 178-185.

Whitfield, C. and Roberts, I.S. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. **31:** 1307-1319.

Whitfield, C. and Valvano, M.A. 1993. Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. Adv. Microb. Physiol. **35:** 135-246.

Williams, A.G. and Wimpenny, J.W.T. 1980. Extracellular polysaccharide biosynthesis by *Pseudomonas* NCIB 11264. Studies on precursor-forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J. Gen. Microbiol. **116:** 133-141.

Wright, A. and Kanegasaki, S. 1971. Molecular aspects of lipopolysaccharides. Physiol. Rev. **51:** 748-784.

Yamamoto, Y., Nunome, T., Yamauchi, R., Kato, K. and Sone, Y. 1995. Structure of an exocellular polysaccharide of *Lactobacillus helveticus* TN-4, a spontaneous mutant strain of *Lactobacillus helveticus* TY1-2. Carbohydr. Res. **275:** 319-332.

Yokoi, H., Watanabe, T. and Fujii, Y. 1990. Isolation and characterization of polysaccharide-producing bacteria from kefir grains. J. Dairy Sci. **73:** 1684-1689.

Chapter 2

Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its synthesis

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ABSTRACT

Exopolysaccharide (EPS) production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 was found to be most efficient with glucose as a substrate. The optimal temperature and pH for EPS synthesis were 25°C and pH 5.8, respectively. EPS production could not be identified as a stress response: increased oxygen tension and reduced water activity negatively affected both growth and EPS synthesis. It is often assumed that there is a competition between growth and EPS formation. Within the range of 0.5 and 0.1 h^{-1}, reducing the growth rate indeed resulted in an increase of the specific EPS production but the polymer formation decreased again at even lower growth rates. Most of the applied fermentation conditions influenced both growth and EPS formation. As the growth rate itself also influenced EPS formation, we studied the linking between growth and EPS synthesis. Interestingly, EPS production was not strictly coupled to growth. Significant *de novo* synthesis of EPS was observed in non-growing cultures. Consequently, the influence of different culture conditions on EPS production could be studied independent of growth.

INTRODUCTION

Exopolysaccharides (EPS) derived from bacterial fermentation, such as xanthan, provide an important source of biothickeners (Sutherland, 1998). Xanthan is already widely used by the food industry but has the disadvantage that it is produced by *Xanthomonas campestris,* a non-GRAS organism (not Generally Regarded As Safe) (de Vuyst *et al.*, 1998). The food industry is interested in EPS-producing food grade organisms such as lactic acid bacteria. EPS produced by lactic acid bacteria can not only be applied as natural additives but can also be produced *in situ.* Without the presence of mucoid lactococci in Scandinavian fermented milk (långfil and viili) for example, these products would lose their characteristic ropy consistency (Cerning, 1990).

EPS synthesised by lactic acid bacteria can be divided into two groups. The first group is composed of homopolysaccharides that are produced extracellularly utilising sucrose as a specific donor. The second group is a heterogeneous group of homo- and heteropolysaccharides produced by mesophilic and thermophilic dairy lactic acid bacteria. EPS produced by lactococci belong to the latter group of polymers that are composed of some hundreds to several thousands repeating units. These intracellularly formed repeating units vary in size from di- to heptasaccharides and can also contain non-sugar molecules (Cerning, 1995). The repeating unit of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 is composed of glucose, galactose, rhamnose and phosphate in a ratio of 2:2:1:1 (van Casteren *et al.*, 1998). This EPS is similar to that produced by *Lactococcus lactis* SBT 0495 (Nakajima *et al.*, 1992), although both strains are not identical.

Most ropy bacteria produce EPS under all conditions but the quantities and the composition of EPS are affected by fermentation conditions and medium composition (Sutherland, 1972). This study focuses on the optimisation of the EPS formation by *Lactococcus lactis* NIZO B40. This strain was chosen as the producing organism because of the promising rheological characteristics of its EPS (Tuinier *et al.*, 1999). Furthermore, the genes necessary for EPS production by lactococci are plasmid encoded and the *eps* gene cluster of strain NIZO B40 is known (van Kranenburg *et al.*, 1997). The involvement of plasmid encoded genes makes it relatively easy to transfer the ability to produce EPS to other strains. This is very useful when studying the biosynthesis of EPS and for metabolic engineering of EPS yield and composition in order to achieve an efficient production of polysaccharides with desired properties.

In order to optimise the production of EPS by strain NIZO B40 we studied the influence of the carbon source, culture conditions, growth rate and stress conditions on the yield and composition of EPS. Furthermore, we investigated the relationship between growth and EPS biosynthesis and we developed a production system with non-growing bacteria to study the influence of different culture conditions on EPS production independent of growth.

MATERIALS AND METHODS

Bacterial strain and media

Lactococcus lactis subsp. *cremoris* NIZO B40 was isolated from Scandinavian ropy milk (van Kranenburg *et al.*, 1997). Stock cultures were maintained in litmus milk and stored at -40 °C. The influence of different sugar sources was studied in M17-broth (Terzaghi and Sandine, 1975). All pH-controlled fermentations were performed in a chemically defined medium (CDM) based on media described earlier (Exterkate, 1979; Otto *et al.*, 1983). The medium contained per litre: 60 g glucose (5 g for glucose-limited growth), 0.6 g $(NH_4)_3$-citrate, 3 g K_2HPO_4, 3 g KH_2PO_4, 1 g Na-acetate, 0.25 g tyrosine, 10 ml nucleotide solution (Otto *et al.*, 1983), 5 ml ascorbic acid solution (0.1 g ml^{-1}), 10 ml trace-element solution, 10 ml vitamin solution and 100 ml amino acid solution (Poolman and Konings, 1988; 10 x concentrated). The medium (pH 6.5) was autoclaved (15 min, 121°C) before the solutions were added. The vitamin solution (pH 6.5) was previously described (Otto *et al.*, 1983) except that 250 mg biotin was added per litre. The trace-element solution contained per litre: 20 g $MgCl_2.3H_2O$, 5 g $CaCl_2.3H_2O$, 1.6 g $MnCl_2.4H_2O$, 0.5 g $FeCl_2.4H_2O$, 0.5 g $ZnSO_4.7H_2O$, 0.3 g $FeCl_3$, 0.25 g $CuSO_4.5H_2O$, 0.25 g $CoSO_4.7H_2O$ and 0.25 g $(NH_4)_6(MO_7)_{24}$. This solution was heated (20 min, 100°C); all the other solutions were sterilised by filtration (0.2 µm). Stock solutions were kept at -40°C but the nucleotide and ascorbic acid solutions were freshly prepared.

Culture conditions and analysis of cell growth

Fermentations were performed at least in duplicate in a 1-litre dished bottom fermenter with a H/D ration of 1.5 (Applikon, The Netherlands) filled with 500 ml of CDM at a constant temperature of 15, 20, 25, 30 or 35°C. An inoculum (1%) of an overnight culture at 30°C was used to start the fermentation. The pH was kept at a certain value (5.3, 5.8, 6.3 or 6.8) after an initial decrease from pH 6.5. Examining a pH of 6.8 was realised by adding NaOH to the culture medium before inoculation. Nitrogen gas was led over the culture medium (30 ml min^{-1}) and agitation speed was at 150 rpm. The pH was controlled by automatic titration with 2 M NaOH. Fermentations were ended when titration of NaOH had stopped. Cell growth was determined by measuring the optical density of the culture fluid at 600 nm (OD_{600}). One OD_{600}-unit corresponded to 375 mg l^{-1} dry mass of cells. Continuous cultures were performed at 30°C and pH 5.8. The liquid volume in the fermenter was kept constant by using a pump controlled by a volume sensor. For these chemostat cultures samples were taken during steady state when at least five fermenter volumes had flown through the system. Residual sugars, organic acids and ethanol were quantified by HPLC.

EPS isolation, quantification and characterisation

For a better comparison of the influence of culture conditions on EPS synthesis we determined the total amount of EPS at the end of an experiment. This included the polymers that were slightly attached to the cells, which might have been growing chains of unfinished EPS. Loosely associated EPS could be liberated by heating the cell suspensions for 3 h at 50°C prior to centrifugation. Although for many bacteria it is known that EPS yields decrease upon prolonged fermentation (de Vuyst *et al.*, 1998), this is not the case for EPS produced by strain NIZO B40. Samples of (heated) culture broth were centrifuged (13,000 x *g*, 10 min, 4°C) and the supernatant fluid was filtered (0.45 μm) without losses. The concentration of EPS in the filtrate was measured in duplicate by gel permeation chromatography (GPC) using dextran (350-500 kg mol^{-1}) as standard. The GPC-system consisted of a sample injector, an isocratic pump, a pre column (Pro-Gel TSK Guard), a column (Pro-Gel TSK G600 PW), a refractive index (RI) detector and an UV-detector (280 nm). As mobile phase 0.1 M NaNO3 was used at a flow rate of 0.6 ml min^{-1}. The concentration of EPS was determined by integration (Spectra-Physics, SP4270) of the area under the RI peak. The standard deviation of this method was 2%. The UV-detector was used to make sure that the EPS peaks were not contaminated with proteins or peptides.

The GPC-EPS peaks were collected and concentrated by ultrafiltration for characterisation of the EPS. Concentrated EPS samples were hydrolysed in 4 M HCl for 30 min at 100°C and dried under vacuum. The monomeric sugar composition of EPS after hydrolysis was determined by HPLC (van Riel and Olieman, 1991). Under these conditions the monosaccharides glucose, galactose, rhamnose, mannose and fructose could be detected as well as the aminosugars glucosamine and galactosamine.

Determination of the percentage of non-producing mutants

Appropriate dilutions of culture medium were plated on GM17-agar and incubated at 30°C for 24 h. Fifty colonies were randomly picked from the plates and transferred to litmus milk with 0.5% yeast extract and 1% glucose. After an incubation of 24 h at 30°C the percentage of tubes with slime formation was determined.

RESULTS AND DISCUSSION

Effect of carbon source and culture conditions

To determine the most suitable carbon source for EPS production, *Lactococcus lactis* subsp. *cremoris* NIZO B40 was grown in M17-broth supplemented with 2% of carbohydrate at 20°C. Samples for analysis of EPS concentration were taken after growth had ceased. During these experiments the pH decreased from 6.8 to 4.5-4.6. On all substrates an OD_{600} of about 2.5 was obtained. The energy sources that were tested were all fermentable carbon

sources. Table 2.1 shows that glucose was the best sugar source for EPS synthesis. The difference in EPS production with glucose and galactose as sugar sources was small but significant. Under pH controlled conditions EPS production was considerably lower with galactose than with glucose as the source of sugar (200 versus 490 mg l^{-1}). With fructose as a source of energy only a minor amount of EPS was produced.

Table 2.1

Effect of carbon source on EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 at 20°C under acidifying conditions.

Carbon source (20 g l^{-1})	EPS (mg l^{-1})
Fructose	<10
Galactose	85
Glucose	94
Lactose	27
Mannose	23
N-acetylglucosamine	56

Table 2.2

Influence of pH and temperature on growth and EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 in CDM with 6% of glucose.[a]

Temperature (°C)	pH	μ_{max}[b] (h^{-1})	OD_{600}	total EPS (mg l^{-1})	specific EPS production (mg EPS/OD_{600}-unit)
30	5.3	0.3	4.6	350	76
30	5.8	0.6	7.1	490	69
30	6.3	0.7	7.4	420	57
30	6.8	0.6	7.1	305	43
15	5.8	0.1	5.5	410	75
25	5.8	0.4	6.4	520	81
30	5.8	0.6	7.1	490	69
35[c]	5.8	0.2	2.5	110	44

[a] All fermentations were carried out in duplicate and the results were within an error range of 10%.

[b] μ_{max} was determined at the moment that the desired pH was reached by linear regression of the $\ln(OD_{600})$ *vs* the time (h).

[c] The residual glucose concentration at the end of this experiment was 25 g l^{-1}.

A chemically defined medium (CDM) supplemented with 6% of glucose was used to find the optimal fermentation parameters for EPS production. The fermentations were performed under pH control to increase the differences between the production yields at the various fermentation conditions because under pH control the cell densities and hence the EPS concentrations increased.

The influence of pH on growth and EPS formation was studied at 30°C (Table 2.2). The highest cell density was reached at pH 6.3 while the total and specific EPS production were highest at, respectively, pH 5.8 and 5.3. The effect of growth temperature was examined at pH 5.8, the optimal pH for EPS production. At 15°C traces of acetic acid (0.4 g l^{-1}) were produced. The highest total concentration of EPS was found at a temperature of 25°C. At the optimal growth temperature of 30°C total EPS biosynthesis was slightly lower (Table 2.2). A temperature of 30°C was continued to be used as cultivation temperature because μ_{max} was higher at this temperature. The course of biomass formation and concentration of EPS in the broth during a standard fermentation at 30°C and pH 5.8 is shown in Fig. 2.1. During this cultivation all of the glucose was consumed and EPS and lactic acid were the only end products. The concentration of EPS in the broth was 460 mg l^{-1} and 30 mg l^{-1} of EPS was associated with the cells at the end of the experiment.

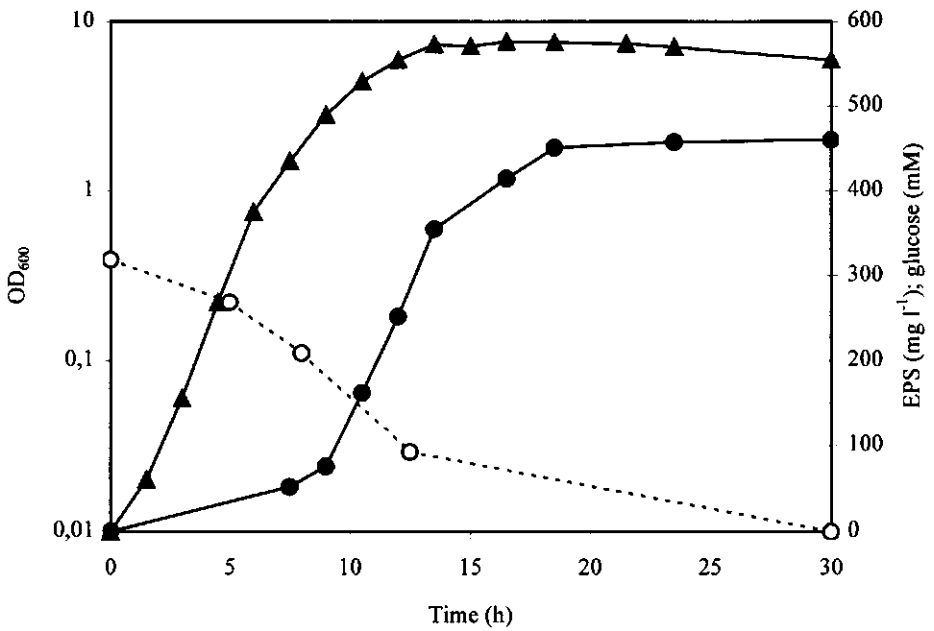

Figure 2.1

Growth (OD_{600}; ▲) and production of EPS by *Lactococcus lactis* subsp. *cremoris* NIZO B40 in a chemically defined medium with 6% of glucose at 30°C and pH 5.8; EPS concentration in the medium (●), Residual glucose concentration (○).

Increasing or decreasing the shear forces by varying the agitation speed from 50 to 800 rpm did not influence growth or EPS production. Total EPS synthesis was also not affected when the titrant NaOH was replaced by NH_4OH, but a much higher percentage of EPS was bound to the cells in this case: 37% at the end of the experiment. Obviously, charge interactions play an important role in the association of EPS with the bacteria.

Influence of stress conditions

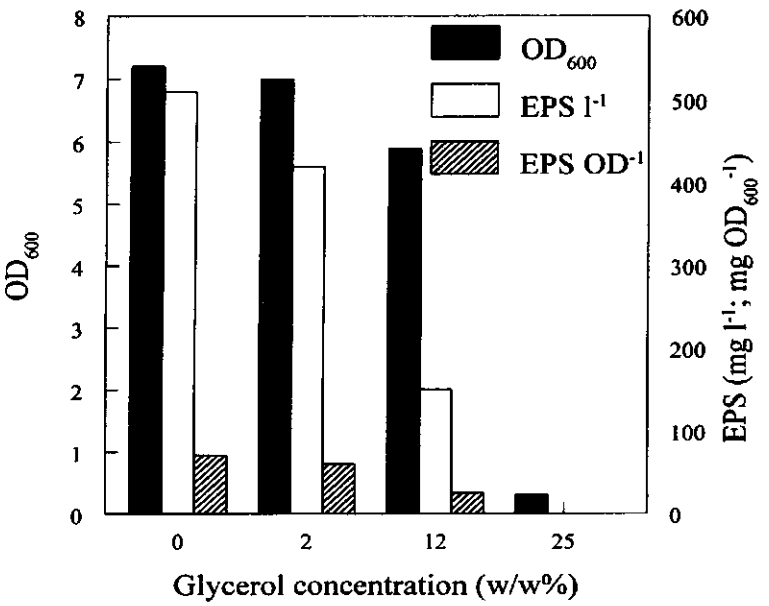

Figure 2.2

Influence of glycerol on growth and EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 in CDM with 6% glucose at pH 5.8 and 30°C.

EPS is often thought to have a protective function (Cerning, 1990). For that reason it was examined if EPS formation could be further optimised by exposing the bacteria to stress conditions such as high osmolarity and high oxygen tension. The maximum concentrations of NaCl, sucrose and glycerol in the medium still allowing growth of strain NIZO B40 were 4, 35 and 25%, respectively. The addition of these water activity-reducing components negatively influenced growth. The lag phase increased, the growth rate decreased and the final cell density decreased when the concentrations of NaCl, sucrose or glycerol were raised. The total and specific EPS production were also reduced when the water activity was lowered

(Fig. 2.2). Comparable results were found in case of increasing the oxygen concentration to an 80% air saturation level by purging air through the CDM (0.5 l min^{-1}) at 30°C and pH 5.8. The final cell density decreased to an OD_{600} of 5.5 and 330 mg l^{-1} of EPS was produced. De Vuyst *et al.* (1998) found comparable results for *S. thermophilus* LY03 in case of increasing the oxygen concentration. It seemed that low water activity or high oxygen tension could not be used as tools to increase the EPS production.

Influence of growth rate

Isoprenoid carrier lipids are involved in biosynthesis of cell wall components such as lipopolysaccharide, teichoic acid, peptidoglycan as well as biosynthesis of EPS (Sutherland, 1982). Due to a competition for these lipids it is often expected that culture conditions resulting in reduced growth rates such as low temperatures could increase EPS production (Cerning, 1995). Decreasing the culture temperature from 30 to 25°C resulted in an increase of the specific EPS production with 17% for strain NIZO B40 (Table 2.2). Cultivation at temperatures below 25°C did not result in any further increase of the specific EPS formation as was found for *Lactobacillus rhamnosus* and *Lactobacillus sake* (van den Berg *et al.*, 1995; Gamar *et al.*, 1997). Nevertheless, the only correct way to study the influence of growth rate on EPS production is in a chemostat. EPS formation by strain NIZO B40 is encoded on a plasmid (van Kranenburg *et al.*, 1997). For that reason it was necessary to determine the stability of this plasmid before the influence of different growth rates on EPS production could be studied in continuous cultures. Grown under glucose limitation in CDM at pH 5.8, 30°C and a dilution rate (D) of 0.2 h^{-1}, EPS synthesis was stable for about 50 cell divisions. Obviously, non-EPS forming mutants develop with a very low frequency and as they emerge, they quickly take over the rest of the culture. Anyway, the EPS-producing trait of strain NIZO B40 was stable enough to perform experiments in continuous cultures.

Table 2.3

Influence of dilution rate (D) on EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 in CDM with 0.5% glucose at pH 5.8 and 30°C.*

D (h^{-1})	OD_{600}	EPS (mg l^{-1})	specific EPS production (mg EPS/OD_{600}-unit)
0.05	1.75	75	43
0.10	1.93	108	56
0.20	1.98	102	52
0.30	2.06	89	43
0.50	2.02	82	40

* All fermentations were carried out at least in duplicate and the results were within an error range of 10%.

Table 2.3 illustrates the concentration of EPS in the broth during steady state of glucose-limited cultures at different dilution rates. EPS production was stable throughout all the fermentations. Under steady state conditions no glucose could be detected in the culture fluid. The specific EPS production was highest at a D of 0.10 h^{-1} but this was still low compared to the batch fermentation shown in Fig. 2.1. The efficiency of EPS production (mg of EPS produced per gram of glucose consumed) was however about two times higher in this case. Apparently the concentration of glucose is very important for the efficiency of the conversion of glucose to EPS and biomass.

Thus growth rate influences EPS formation although the lowest growth rate did not result in the highest productivity. This is consistent with the experiments at different culture temperatures but not in accordance with the theory described above. At a D of 0.05 h^{-1} EPS production was possibly not determined by the availability of lipid intermediates but by other limiting factors. Growth and EPS synthesis do not only share the use of lipid intermediates but are both also dependent on substrate uptake and the availability of sugar nucleotides (Sutherland, 1985) as well as energy supply (Sutherland, 1982). A growth rate of 0.05 h^{-1} resulted in a lower final cell density than growth rates of 0.10-0.50 h^{-1}. This is probably caused by the fact that non-growth associated ATP consumption is relatively high at low growth rates (Tempest, 1978). At low growth rates energy supply could also be the limiting factor for EPS production. However, when the rate of formation of cell material is limited by the availability of ATP a shift from homolactic to mixed acid fermentation would be expected (Cocaign-Bousquet *et al.*, 1996). In the glucose-limited cultures of strain NIZO B40 there was little diversion towards mixed acid fermentation at growth rates of 0.05 and 0.10 h^{-1}: formic acid, acetic acid and ethanol accounted for respectively 7 and 6% of the total product formation.

Uncoupling of growth and EPS production

The culture conditions described above not only affected the EPS production but influenced growth (rate) also (Table 2.2). From Table 2.3 it is clear that the growth rate itself also affects EPS formation. For an accurate study of the influence of culture conditions on EPS synthesis it is essential to maintain normal growth or to study EPS biosynthesis under non-growing conditions. For many Gram-negative bacteria like *Enterobacter aerogenes* (Sutherland, 1985), *Pseudomonas* (Williams and Wimpenny, 1980) and *Rhizobium leguminosarum* (Breedveld *et al.*, 1993) it is known that non-growing cells can be used for the production of exopolysaccharides. We investigated if this principle could also be applied for *Lactococcus lactis* subsp. *cremoris* NIZO B40.

Fig. 2.1 shows that EPS production by strain NIZO B40 starts at the exponential phase but continues during the early stationary phase as was also found for other lactic acid bacteria (Kojic *et al.*, 1992; Bouzar *et al.*, 1996). This could indicate that growth and EPS production

are not strictly linked. Another indication for a possible uncoupling is the fact that optimal temperature and pH for EPS production and growth are not the same for strain NIZO B40. By studying EPS synthesis of washed cells in medium without a nitrogen source it was clearly demonstrated that EPS production by strain NIZO B40 is not strictly linked to growth. Growth was not possible in this nitrogen-free medium but the cells were still metabolically active and both lactic acid and EPS were produced (Fig. 2.3).

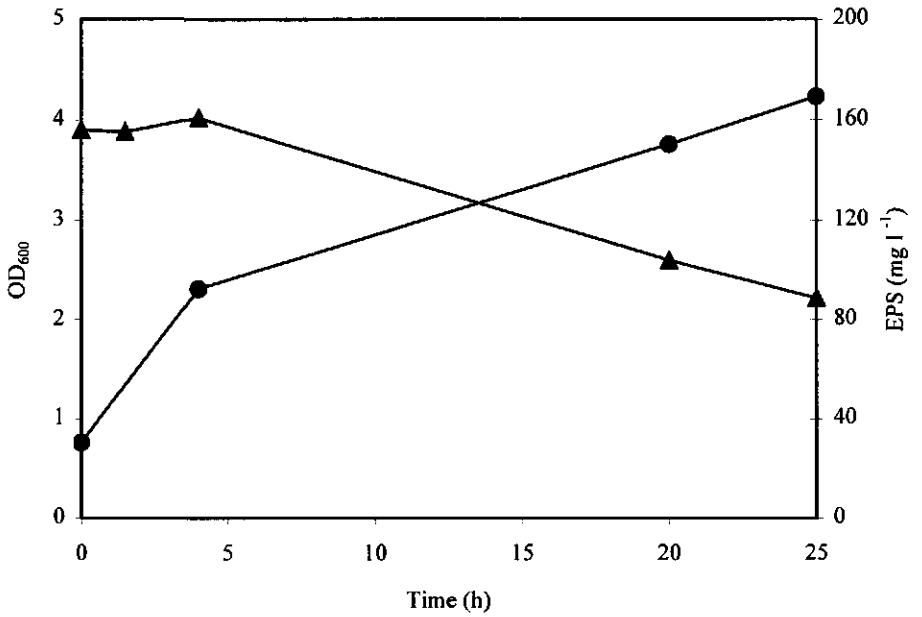

Figure 2.3

OD_{600} (▲) and EPS production (●) of washed cells of *Lactococcus lactis* subsp. *cremoris* NIZO B40 in CDM without amino acids at pH 5.8 and 30°C.

To study the influence of different culture conditions on EPS synthesis independent of growth, EPS production by non-growing cultures of strain NIZO B40 was investigated. This strain was cultured in CDM with 6% of glucose at 30°C and pH 5.8. At the moment that an OD_{600} of 2.5 was reached, chloramphenicol (10 μg ml^{-1}) was added to the culture medium and the temperature was changed to the temperature under investigation. After addition of chloramphenicol the OD_{600} of the cultures increased till 3.0. The amount of EPS present at that moment was subtracted from the final total EPS concentration to determine the EPS formation at the different conditions. Chloramphenicol prevents growth by inhibiting the peptidyl transferase activity of the 50S ribosomal subunit. For these experiments it is therefore assumed that all the enzymes necessary for EPS biosynthesis are present in sufficient amounts and are stable throughout the experiments.

At 25°C non-growing cultures of NIZO B40 produced the highest amount of EPS: 285 mg l^{-1}. At 20, 30 and 35°C the EPS production was respectively, 200, 230 and 150 mg l^{-1}. In these experiments, as was also found for growing cultures, a temperature of 35°C resulted in a markedly decreased EPS formation. The influence of water activity on EPS synthesis by non-growing cultures was comparable with that of growing cultures: the EPS production decreased when the concentration of glycerol in the medium was raised.

EPS production by lactic acid bacteria is often regarded to be strictly growth-associated (van den Berg *et al.*, 1995; Grobben *et al.*, 1996; Gamar *et al.*, 1997; de Vuyst *et al.*, 1998). We demonstrated that EPS synthesis by *Lactococcus lactis* NIZO B40 is not strictly linked to growth. A biotechnological application of EPS production by these non-growing cultures could be the use of *Lactococcus lactis* as a cell factory for the continuous production of biothickeners.

Composition of EPS

For most bacteria the composition of EPS is independent of the composition of the medium (Sutherland, 1972). However, the sugar composition of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Grobben *et al.*, 1996) and *Lactobacillus casei* (Kojic *et al.*, 1992) was reported to be dependent on medium composition. The culture conditions, carbon source and growth rate did not influence the sugar composition of EPS produced by strain NIZO B40. Glucose, galactose and rhamnose were found in a ratio of 2.2:1.3:1.0. This is not completely in accordance with the proposed structure, but the amount of galactose is probably underrated by the presence of phosphate (van Casteren *et al.*, 1998).

The molecular mass of the EPS produced was significantly reduced under certain growth conditions (results not shown). As the molecular mass is very important for the viscosifying properties of the EPS, the effect of culture conditions on the molecular mass of EPS is currently being investigated. Furthermore, the regulation of EPS production by the source of sugar and the physiological role of B40 EPS will be studied.

ACKNOWLEDGEMENTS

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REFERENCES

Bouzar, F., Cerning, J. and Desmazeaud, M. 1996. Exopolysaccharide production in milk by *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 1187 and by two colonial variants. J. Dairy Sci. **79:** 205-211.

Breedveld, M.W., Zevenhuizen, L.P.T.M., Canter Cremers, H.C.J. and Zehnder, A.J.B. 1993. Influence of growth conditions on production of capsular and extracellular polysaccharides by *Rhizobium leguminosarum*. Antonie van Leeuwenhoek **64:** 1-8.

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87:** 113-130.

Cerning, J. 1995. Production of exopolysaccharides by lactic acid bacteria and dairy propionibacteria. Lait **75:** 463-472.

Cocaign-Bousquet, M., Garrigues, C., Loubiere, P. and Lindley, N.D. 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie van Leeuwenhoek, **70:** 253-267.

de Vuyst, L., Vanderveken, F., van de Ven, S. and Degeest, B. 1998. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. J. Appl. Microbiol. **84:** 1059-1068.

Exterkate, F.A. 1979. Accumulation of proteinase in the cell wall of *Streptococcus cremoris* strain AM1 and regulation of its production. Arch. Microbiol. **120:** 247-254.

Gamar, L., Blondeau, K. and Simonet, J.-M. 1997. Physiological approach to extracellular polysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. **83:** 281-287.

Grobben, G.J., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1996. Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. Appl. Microbiol. Biotechnol. **46:** 279-284.

Kojic, M., Vujcic, M., Banina, A., Cocconcelli, P., Cerning, J. and Topisirovic, L. 1992. Analysis of exopolysaccharide production by *Lactobacillus casei* CG11, isolated from cheese. Appl. Environ. Microbiol. **58:** 4086-4088.

Nakajima, H., Hirota, T., Toba, T., Itoh, T. and Adachi, S. 1992. Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. Carbohydr. Res. **224:** 245-253.

Otto, R., ten Brink, B., Veldkamp, H. and Konings, W.N. 1983. The relation between growth rate and electrochemical proton gradient of *Streptococcus cremoris*. FEMS Microbiol. Lett. **16:** 69-74.

Poolman, B. and Konings, W.N. 1998. Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. J. Bacteriol. **170:** 700-707.

Sutherland, I.W. 1972. Bacterial exopolysaccharides. Adv. Microb. Physiol. **8:** 143-213.

Sutherland, I.W. 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23:** 79-150.

Sutherland, I.W. 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Ann. Rev. Microbiol. **39:** 243-270.

Sutherland, I.W. 1998. Novel and established applications of microbial polysaccharides. Tibtech **16:** 41-46.

Tempest, D.W. 1978. The biochemical significance of microbial growth yields: a reassessment. Tibs **3:** 180-184.

Terzaghi, B.E. and Sandine, W.E. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:** 807-813.

van Casteren, W.H.M., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1998. Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40. Carbohydr. Polym. **37:** 123-130.

van den Berg, D.J.C., Robijn, G.W., Janssen, A.C., Giuseppin, M.L.F., Vreeker, R., Kamerling, J.P., Vliegenthart, J.F.G., Ledeboer, A.M. and Verrips, C.T. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. Appl. Environ. Microbiol. **61:** 2840-2844.

van Kranenburg, R., Marugg, J.D., Van Swam, I.I., Willem, N.J. and De Vos, W.M. 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lacotococcus lactis*. Mol. Microbiol. **24**: 387-397.

van Riel, J. and Olieman, C. 1991. Selectivity control in the anion-exchange chromatographic determination of saccharides in dairy products using pulsed amperometric detection. Carbohydr. Res. **215:** 39-46.

Williams, A.G. and Wimpenny, J.W.T. 1980. Extracellular polysaccharide biosynthesis by *Pseudomonas* NCIB 11264. Studies on precursor-forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J. Gen. Microbiol. **116:** 133-141.

Chapter 3

Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures

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ABSTRACT

The type of substrate limitation had a remarkable influence on the molecular mass of exopolysaccharides (EPS) produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891. Under glucose/energy limitation the molecular mass was much smaller than under leucine or phosphate limitation, resulting in a marked decrease of the intrinsic viscosity of this EPS. The sugar composition of EPS produced by both strains, and the phosphate content of EPS produced by NIZO B40, were not affected by the type of nutrient limitation. Both strains produced comparable amounts of EPS under leucine and glucose limitation, but the efficiency of EPS production was highest under glucose limitation. The EPS yields of the phosphorylated B40 EPS as well as the unphosphorylated B891 EPS were reduced, with about 40% under conditions of phosphate limitation.

Introduction

Polymers are commonly used as thickening, stabilising, emulsifying or gelling agents for various food and non-food applications. Since early times, polysaccharides of plant origin have been employed for these purposes, but the practical use of exopolysaccharides (EPS) derived by microbial fermentation dates from more recent years (Fett *et al.*, 1996). The first industrial polysaccharide of microbial origin used for food applications was xanthan, produced by *Xanthomonas campestris*. The United States Food and Drug Administration (FDA) approved this EPS for general food use in 1969. Other commercially produced bacterial EPSs that are used as food additives to date are gellan and cellulose (Fett *et al.*, 1996).

Nowadays, a lot of effort is put in the selection of new microbial strains and optimisation of culture conditions to achieve higher yields of those EPSs already commercially successful. Furthermore, there is a considerable interest in finding new EPSs that are suitable for special applications, or that have a potential industrial relevance, either by applying different culture conditions or by using novel bacterial strains (Crescenzi, 1995). EPSs produced by lactic acid bacteria play a role in the rheology and texture of fermented milks (Cerning, 1990) and could also provide a new source of safe additives for use in various food products (van den Berg *et al.*, 1995). The physical properties of EPS, such as the rheology and the phase behaviour, depend on several factors, including sugar composition, type of sugar linkages, the presence of organic or inorganic substituents, the degree of polymerisation and the length of the side chains (Sutherland, 1994).

In this study, the production of EPS by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891 was investigated. The primary structures of the repeating unit of B40 EPS (van Kranenburg *et al.*, 1997; van Casteren *et al.*, 1998; van Casteren *et al.*, 1999) and of B891 EPS (van Casteren *et al.*, 2000) are shown in Fig. 3.1. The EPS produced by strain NIZO B40 consists of pentasaccharide repeating units containing glucose, galactose, rhamnose and phosphate in a ratio of 2:2:1:1. The thickening properties of this B40 EPS, which is negatively charged above pH 2 due to the presence of phosphate groups, are very promising (Tuinier *et al.*, 1999a). The EPS produced by strain NIZO B891 consists of a pentasaccharide repeating unit containing glucose and galactose in a ratio of 3:2 and appears to be acetylated.

Several studies have reported that the yield (van den Berg *et al.*, 1995; Gamar *et al.*, 1997; Grobben *et al.*, 1998; Looijesteijn and Hugenholtz, 1999; de Vuyst and Degeest, 1999) and composition (Petit *et al.*, 1991; Grobben *et al.*, 1997) of EPS produced by lactic acid bacteria can be influenced by changing the culture conditions or the medium composition. Recently, Faber *et al.* (1998) reported that the repeating units of EPSs produced by *Streptococcus thermophilus* strains Rs and Sts are identical, although the strains differ in the viscosity of their milk cultures probably because of a difference in the molecular masses of their EPSs. The type of nutrient limitation could have an influence on EPS yield or EPS composition.

Furthermore, if the chemical composition of the repeating units or the molecular mass of EPS can be changed by variations in the growth conditions, it would be interesting to study the effect of these changes on the relative physical properties. Therefore, the influence of different substrate limitations on the yield, composition, molecular mass and viscosity of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891 was studied.

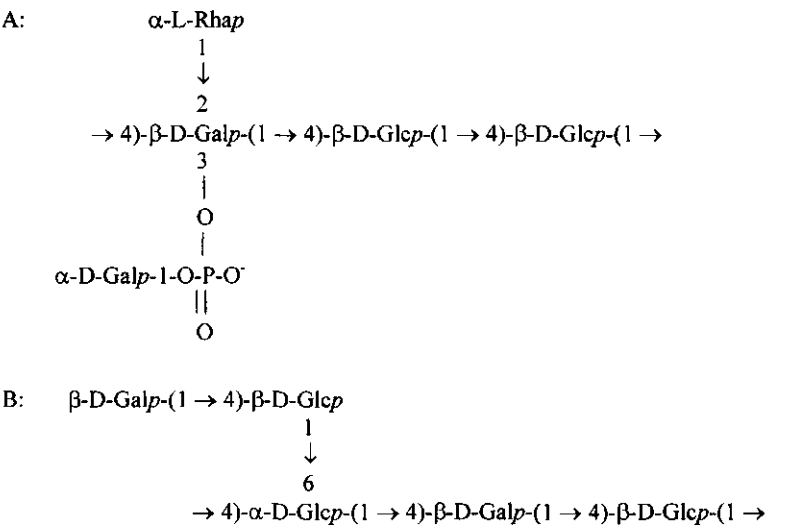

Figure 3.1

Primary structure of the repeating units of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 (A; van Kranenburg *et al.*, 1997; van Casteren *et al.*, 1998; van Casteren *et al.*, 1999) and NIZO B891 after deacetylation (B; van Casteren *et al.*, 2000).

MATERIALS AND METHODS

Micro-organisms and growth conditions

Lactococcus lactis subsp. *cremoris* NIZO B40 (van Kranenburg *et al.*, 1997) and NIZO B891 used in this study are natural EPS-producing strains. The bacteria were stored at –40°C in litmus milk.

Fermentations were performed at 30°C in a 1-litre fermenter filled with 500 ml of a chemically defined medium (CDM) as described previously (Looijesteijn and Hugenholtz, 1999). An inoculum (1%) of an overnight culture at 30°C was used to start the fermentation. The pH was kept at 5.8 by automatic titration with 2 mol l^{-1} NaOH. This pH was chosen because it resulted in the highest concentration of EPS in pH-controlled batch cultures for *Lactococcus lactis* NIZO B40 (Looijesteijn and Hugenholtz, 1999). Nitrogen gas was led over

the culture medium, in which a constant agitation speed of 50 rpm was imposed. Samples from the continuous cultures were taken during steady state when at least five fermenter volumes had passed the system. From that point on about five litres of culture broth were collected for the characterisation of the EPS produced. The concentration of glucose was reduced from 278 to 28 mmol l^{-1} for fermentations under glucose limitation, and in case of leucine limitation the concentration of leucine was reduced from 3.62 to 0.23 mmol l^{-1}. In order to study phosphate-limited growth, the amount of phosphate was reduced to 0.6 mmol l^{-1}, and as phosphate also functions as buffer in the CDM, 50 mmol l^{-1} 2-[N-Morpholino]ethanesulfonic acid (MES) was added under this condition. Addition of MES did not influence growth or EPS production. Substrate limitation was checked by pulsing with the limiting component.

Analysis of cell growth, residual sugars and product formation

Cell growth was determined by measuring the optical density of the culture fluid at 600 nm (OD_{600}). The concentrations of EPS in the culture broth were measured by a combination of refractive index measurement and gelpermeation chromatography using dextran as standard, as described previously (Looijesteijn and Hugenholtz, 1999). Here, specific EPS production is defined as the amount of EPS produced per OD_{600}-unit, and the efficiency of EPS production as the amount of EPS produced per amount of glucose that was consumed. Residual sugars, organic acids and ethanol were quantified by HPLC (Looijesteijn *et al.*, 1999).

Isolation and Purification of EPS

Bacterial cells were removed from the EPS-containing culture broth by centrifugation (10,000 x *g*, 30 min). The resulting supernatant fluid was concentrated by ultrafiltration using a polysulfone membrane with a molar exclusion limit of 50 kDa. The retentate was diafiltrated to remove low molecular impurities. The remaining solution was freeze-dried. The EPSs were further purified by suspending 100 mg powder in 100 ml 80% ethanol containing 0.1% formic acid (Tuinier *et al.*, 1999a). After centrifugation, the EPS pellet was washed twice with 96% ethanol. Finally, the remaining ethanol was evaporated.

Characterisation of EPS

The sugar composition and phosphate content of the EPS were determined as described by van Casteren *et al.* (1998). In order to analyse the sugar composition, purified EPS was pre-hydrolysed in 12 mol l^{-1} H_2SO_4 (1 h at 30°C) and hydrolysed in 1 mol l^{-1} H_2SO_4 (3 h at 100°C), using inositol as internal standard. The released sugars were converted into their alditol acetates and analysed by gas chromatography.

The amount of phosphorus was determined using a method based on colour formation caused by the reduction of phosphomolybdate by ascorbic acid. The EPS solutions and standards were destructed with 72% perchloric acid (20 min at 180°C). A mixture of

sulphuric acid, ammonium molybdate and ascorbic acid in water was added to the resulting destruction fluid. After an incubation of 2 hours at 37°C, the extinction at 820 nm was measured, from which the amount of phosphorous was calculated (Chen *et al.*, 1956).

For the analysis of molar mass, solutions of purified EPS in 0.1 mol l^{-1} $NaNO_3$ were size fractionated (analytically) by gel permeation chromatography using 0.1 mol l^{-1} $NaNO_3$ as eluent at a flow rate of 1 ml min^{-1} (Tuinier *et al.*, 1999a). The fractions were analysed on-line by RI detection to determine the polysaccharide concentration, by UV transmission at 280 nm to check if the solutions were contaminated with proteins, and by multi-angle static light scattering (SLS). SLS yields the molar mass and radius of gyration.

Determination of viscosity

The viscosities of B40 EPS were measured in a 0.1 mol l^{-1} $NaNO_3$ solution at 20°C using an Ubbelohde capillary. An ionic strength of 0.10 mol l^{-1} is a typical value for food products and is high enough to effectively screen the negative charges of the B40 EPS. The kinematic viscosity (υ) was calculated from the flow times through the capillary, taking into account the Hagenbach correction: $\upsilon = k * t_c$, where t_c is the corrected flow time and k is a constant which only depends on the capillary geometry. The dynamic viscosity, η, follows from $\eta = \rho * \upsilon$, where ρ is the solution density. The results will be presented as the dimensionless specific viscosity η_{sp}, which equals $\eta_{sample}/\eta_{solvent} - 1$. At low EPS concentrations, the solution density of the sample is equal to the solution density of the solvent and hence $\eta_{sp} = [t_{c,sample}/t_{c,solvent}] - 1$. The specific viscosity was determined as a function of the EPS concentration, and from the data in the low concentration range the intrinsic viscosity was determined. The intrinsic viscosity is the parameter which characterises the thickening efficiency of a polysaccharide in all concentration regimes.

The zero-shear intrinsic viscosity, $[\eta]_0$, can be calculated from the molar mass (M) and the radius of gyration (R), as found with SLS: $[\eta]_0 = 5/2 * 4\pi/3 * R^3 N_{av}/M$ where N_{av} is the number of Avogadro (Tuinier *et al.*, 1999b).

RESULTS

Influence of growth rate on EPS production

The influence of the growth rate on EPS production was investigated in a continuous culture using either complete CDM with an undefined component as limiting factor, or under conditions of glucose limitation. In complete CDM, the amount of EPS produced by strain NIZO B40 per OD_{600} unit increased when the dilution rate (D) was decreased (Fig. 3.2). For growth under glucose limitation, a slightly different pattern was found. Reduction of the growth rate from 0.5 to 0.1 h^{-1} resulted in an increase of the specific EPS yield, but a further reduction of the D to 0.05 h^{-1} resulted in a decrease of the polymer yield (Fig. 3.2).

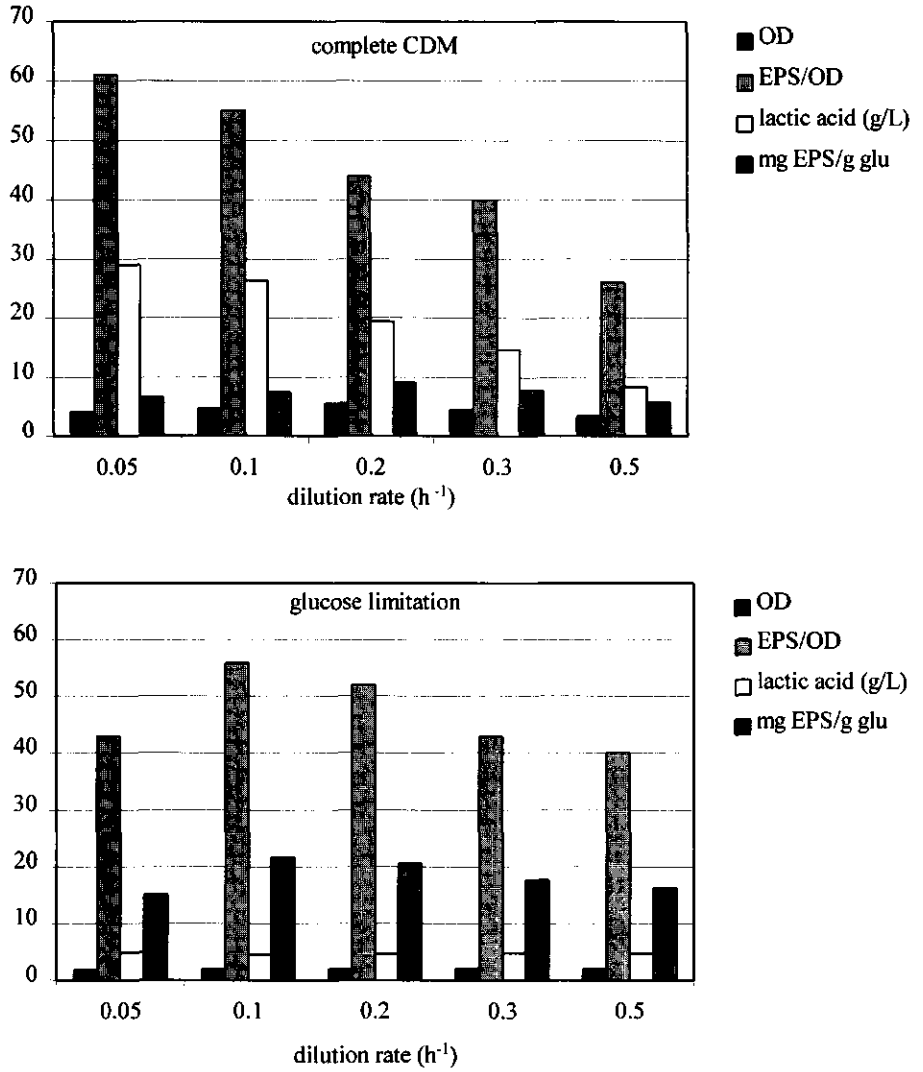

Figure 3.2

Growth and product formation by *Lactococcus lactis* subsp. *cremoris* NIZO B40 in CDM and under glucose limitation at different dilution rates, pH 5.8 and 30°C.

At all growth rates tested, the efficiency of EPS production (mg EPS g glucose consumption^{-1}) was significantly higher under glucose limitation than in complete CDM. Furthermore, Fig. 3.2 shows that the largest production of lactic acid in complete CDM corresponded to the lowest growth rate tested. Especially at low growth rates in this medium,

the energy generation due to the production of lactic acid seems to be completely uncoupled from biomass formation. The molecular mass of the B40 EPS was not influenced by the growth rate.

Influence of various nutrient limitations on EPS yield

In order to improve the EPS yield, the influence of glucose, leucine and phosphate limitation on EPS production by *Lactococcus lactis* NIZO B40 and NIZO B891 was studied in continuous cultures at a D of 0.2 h^{-1}. This dilution rate, which was not the optimal D as determined for strain NIZO B40 in complete CDM and under glucose limitation (Fig. 3.2), was chosen for practical reasons. The concentration of the limiting nutrient was adjusted to give an OD of 1.5-2.0 at 600 nm to enable a good comparison.

Culturing under conditions of glucose limitation provided the highest specific EPS production for *Lactococcus lactis* NIZO B40 and NIZO B891, whereas EPS production was lowest under phosphate limitation (Table 3.1). Glucose was used most efficiently for the production of both EPSs under conditions of glucose limitation (Table 3.1). Strain NIZO B891 produced less EPS than strain NIZO B40 under all substrate limitations. This was the case in batch cultures where NIZO B40 and NIZO B891 produced, respectively, 460 and 140 mg EPS l^{-1} when grown for 26 h, at 30°C and pH 5.8, in CDM with 6% glucose. The efficiency of EPS production in these cultures was, respectively, 8 and 2.5 mg g^{-1} glucose.

The maximum growth rate in the leucine-limited cultures was about 0.3 h^{-1} (not shown). The highest growth rate attainable in glucose-limited cultures was 0.5 h^{-1}. This is in agreement with the findings of Poolman and Konings (1988). They showed that a reduction in the concentration of a single branched amino acid resulted in growth inhibition, whereas this was not the case when the concentrations of all three branched amino acids (leucine, isoleucine and valine) were decreased. To exclude this effect on growth rate, not only were experiments performed in CDM with a reduced concentration of leucine, but also in CDM with decreased concentrations of leucine, isoleucine and valine. This did not result in a higher specific EPS production than under glucose limitation. The EPS yield of NIZO B40 could not be improved by growing this strain in medium with only 10% of all the amino acids of the CDM in continuous cultures, or by limitation of other amino acids than leucine (not shown).

Influence of various substrate limitations on EPS composition

The structures of the repeating units of EPS produced by strains NIZO B40 and NIZO B891 are shown in Fig. 3.1. The chemistry of the repeating units determines the size of a polymer in solution per unit mass. Changing the sugar composition or the phosphate content of the repeating units could thereby affect the rheological properties of the polymer in solution. The number of repeating units polymerised in order to form the polysaccharides is also very important for the determination of the physical characteristics of EPSs (Faber *et al.*, 1998).

Table 3.1

Yield, sugar composition, molar mass and radius of gyration (number averaged) of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891 grown under glucose, leucine and phosphate limitation at pH 5.8, 30°C and D=0.2 h^{-1}.

Strain	Limitation	EPS ($mg\ OD^{-1}$)	EPS ($mg\ EPS\ g\ glu^{-1}$)	Sugar composition (molar ratio)[a]			Phosphate content ($mol\ PO_4/mol\ Glu$)	M_n ($kg\ mol^{-1}$)	r_n (nm)
				rhamnose	galactose	glucose			
NIZO B40	Glucose	52	20.6	1.0	1.4[b]	2.0	0.56	159	32
	Leucine	46	9.6	1.0	1.3	2.0	0.56	480	68
	Phosphate	33	5.7	0.9	1.2	2.0	0.54	601	74
	Complete CDM	44	9.1	0.8	1.2	2.0	ND[c]	ND	ND
NIZO B891	Glucose	30	13.7	traces[d]	2.2	3.0	-	361	39
	Leucine	27	4.2	traces	1.7	3.0	-	836	52
	Phosphate	19	2.6	traces	1.9	3.0	-	881	46
	Complete CDM	15	2.3	ND	ND	ND	-	ND	ND

[a] Based on the chemical structure of the repeating units (Fig. 3.1) glucose is taken as 2.0 (B40 EPS) and 3.0 (B891 EPS)

[b] The relative amount of galactose in B40 EPS is underestimated because of incomplete hydrolysis due to the presence of phosphate (van Casteren *et al.*, 1998)

[c] Not Determined

[d] Since the repeating unit of B891 EPS (Fig. 3.1) does not contain rhamnose, these traces must have been derived from impurities in the analysed EPS samples.

In order to investigate whether changing the culture conditions affects the physical properties of EPS, the effect of various limitations on the polysaccharide composition was studied. None of the limitations resulted in a significantly altered chemistry of the repeating units for either type of EPS (Table 3.1), but the type of nutrient limitation had a huge influence on the molecular mass of the EPSs. Compared with the EPS produced under conditions of phosphate and leucine limitation, the degree of polymerisation of both B40 and B891 EPS was significantly reduced under glucose limitation (Table 3.1). The EPS produced by strain NIZO B891 was more compact than B40 EPS under all limiting conditions.

Viscosity of B40 EPS produced in continuous cultures

The results for the specific viscosity (η_{sp}) of B40 EPS, produced under different substrate limitations as a function of the EPS concentration, are plotted in Fig. 3.3. At low concentrations, there is a linear relationship between EPS concentration [EPS] and specific viscosity, with a slope that equals the intrinsic viscosity [η]: η_{sp} = [EPS] * [η]. The intrinsic viscosity corresponds to the hydrodynamic volume per unit mass, which is an important parameter for the thickening properties of polysaccharides. From the viscosity measurements , intrinsic viscosities of 0.32, 0.74 and 0.85 l g EPS^{-1}, for B40 EPS produced under glucose, leucine and phosphate limitation, respectively, were calculated.

The results obtained with the Ubbelohde measurements cannot be taken as the zero-shear limit $[\eta]_0$, as the shear-rates in capillaries are usually quite high (exceeding 1,000 s^{-1}), but they illustrate the influence of the different culture conditions on the viscosity of the various EPSs. The $[\eta]_0$ can be calculated from the radius of gyration and the molar mass, which were obtained by SLS (Tuinier *et al.*, 1999b). Grown under glucose, leucine and phosphate limitation, the calculated values of $[\eta]_0$ were 1.3, 4.1 and 4.3, respectively, for B40 EPS. Apart from a scaling factor, which is due to the high shear rates, the experimental results agree with the calculated intrinsic viscosities.

DISCUSSION

In CDM, the sugar source is essential for growth as well as EPS production by mucoid lactococci as it provides the energy necessary for both processes. Furthermore, a fraction of the sugar source is used for the biosynthesis of biomass and EPS precursors. Amino acids are not directly involved in EPS biosynthesis but serve as carbon and nitrogen sources which are essential for growth. With this in mind, it could be expected that the amount of EPS produced per cell by *Lactococcus lactis* is lower under glucose limitation than under leucine limitation. Both limitations, however, resulted in comparable specific EPS production based on cell mass by strains NIZO B40 and NIZO B891. This is in direct contrast to reports of other EPS-producing micro-organisms in which higher EPS production was observed under nitrogen-

limited conditions than under carbon-limited conditions (Mengistu *et al.*, 1994; Marshall *et al.*, 1995). For many other bacteria, not only nitrogen but also sulphur and phosphate limitation led to an increase in EPS yield and sometimes, even to a change in EPS composition (Sutherland, 1982; Farrés *et al.*, 1997).

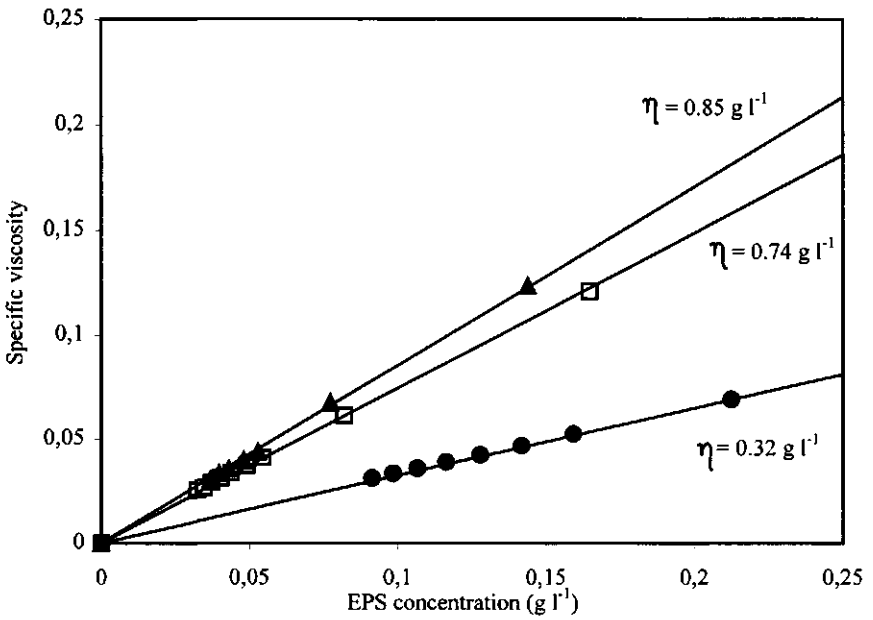

Figure 3.3

Specific viscosity as a function of concentration of EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 under glucose (●), leucine (□) and phospate (▲) limitation at a D of 0.2 h^{-1}, 30°C and pH 5.8.

The quantity of EPS produced per quantity of glucose consumed was highest under glucose limitation. Under phosphate and leucine limitation, both strains produced much more lactic acid than under glucose limitation. This was also observed in the continuous cultures using complete CDM, especially at low growth rates. Under all these conditions, more ATP was generated than was necessary for biomass synthesis. Biosynthesis of biomass and EPS biosynthesis follow roughly the same metabolic pathways. This results in the same metabolic control for EPS production and for growth. The uncoupling of growth and acid production explains the reduction in efficiency of EPS production in the cultures not glucose-limited.

In complete CDM, specific EPS production was highest at the lowest D tested, whereas an optimal D for EPS production was found under conditions of glucose limitation. Production of EPS production and synthesis of cell surface polysaccharides both require isoprenoid lipid

carriers, sugar nucleotides and energy, and competition between the two processes is possible for any of these factors (Sutherland, 1982). Energy supply could have been the limiting factor for EPS production at a D of 0.05 h^{-1} under glucose limitation because of relatively high non-growth associated ATP consumption at low growth rates (Tempest, 1978). At higher growth rates, more intermediates per time unit are needed for the biosynthesis of cell surface polysaccharides, and the intermediates are apparently used in favour of the synthesis of these polysaccharides; this may explain the reduction of the EPS yields at higher growth rates.

Not only the concentration but also the structure of the EPS is important for its thickening effect. There are different approaches to changing the structures of EPSs. The functional properties of the EPS produced could be modified by influencing the growth conditions or by genetic engineering, and after production of EPSs, their structures could be altered via chemical or enzymatic treatment (van Casteren *et al.*, 1998; van Casteren *et al.*, 1999). Growing *Lactococcus lactis* NIZO B40 under different culture conditions and sugar sources did not result in an altered sugar composition of the EPS produced (Looijesteijn and Hugenholtz, 1999). The type of nutrient limitation did not influence the chemistry of the repeating units of the EPSs produced by strains NIZO B40 and NIZO B891, but it had an enormous effect on the molecular masses of the EPSs. Compared with leucine and phosphate limitation, the molecular masses of the B40 and B891 EPSs were strongly reduced under glucose limitation. The B40 EPS molecule was less compact than the B891 EPS molecule under all three limitations. At equal molecular masses, the B40 EPS would probably have better thickening properties than the B891 EPS.

There are only a few reports on the relation between growth conditions and the molecular mass of the EPS. Sutherland (1982) reported that *Xanthomonas juglandis* produced longer EPS molecules at lower dilution rates. For *Lactococcus lactis* subsp. *cremoris* LC330, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 and *Streptococcus thermophilus* LY03, which produce both a high and a low molecular mass EPS, a change in the ratio between the low and high molecular mass EPSs was demonstrated when the culture conditions or the medium compositions were changed. (Marshall *et al.*, 1995; Grobben *et al.*, 1997; Degeest and de Vuyst, 1999).

As far as is know, this is the first demonstration that the molecular mass of an EPS produced by lactic acid bacteria can actually be changed by the culture conditions. A speculative explanation for this phenomenon could be that one of the genes of the *eps* gene clusters of both NIZO B40 and NIZO B891 is *epsB* (van Kranenburg *et al.*, 1997; van Kranenburg *et al.*, 1999). The gene product EpsB contains an ATP binding domain and is homologous to ExoP-like proteins involved in chain-length determination (van Kranenburg *et al.*, 1997). The intracellular concentration of ATP is reduced in glucose-limited cultures compared with leucine-limited cultures (Otto, 1984). This difference in the intracellular ATP concentration could have an effect on ATP-dependent chain-length control.

The reduced viscosifying properties of the EPS produced by strain NIZO B40 might enable the use of this low molecular mass EPS as bodying agent. It has a low biodegradability (Ruijssenaars *et al.*, 2000) and could therefore be used as a non-digestible food fraction in those functional food products where an increase in viscosity is undesirable.

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REFERENCES

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87:** 113-130.

Chen, P.S. Jr., Toribara, T.Y. and Warner, H. 1956. Microdetermination of phosphorus. Anal. Chem. **28:** 1756-1758.

Crescenzi, V. 1995. Microbial Polysaccharides of applied interest: ongoing research activities in Europe. Biotechnol. Prog. **11:** 251-259.

Degeest, B. and de Vuyst, L. 1999. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by *Streptococcus thermophilus* LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. Appl. Environ. Microbiol. **65:** 2863-2870.

de Vuyst, L. and Degeest, B. 1999. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev. **23:** 153-177.

Faber, E.J., Zoon, P., Kamerling, J.P. and Vliegenthart, J.F.G. 1998. The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures. Carbohydr. Res. **310:**269-276.

Farrés, J., Caminal, G. and López-Santín, J. 1997. Influence of phosphate on rhamnose-containing exopolysaccharide rheology and production by *Klebsiella* I-714. Appl. Microbiol. Biotechnol. **48:** 522-527.

Fett, W.F., Osman, S.F., Fishman, M.L. and Ayyad, K. 1996. Biopolymers from fermentation. In: *Agricultural materials as renewable resources: nonfood and industrial applications*, ACS Symposium Series No. 647, ed. Fuller, E.G., McKeon, T.A. and Bills, D.D., pp. 76-87, Washington.

Gamar, L., Blondeau, K. and Simonet, J.-M. 1997. Physiological approach to extracellular polysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. **83:** 281-287.

Grobben, G.J., van Casteren, W.H.M., Schols, H.A., Oosterveld, A., Sala, G., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1997. Analysis of the exopolysaccharides produced by

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Appl. Microbiol. Biotechnol. **48:** 516-521.

Grobben, G.J., Chin-Joe, I., Kitzen, V.A., Boels, I.C., Boer, F., Sikkema, J., Smith, M.R. and de Bont, J.A.M. 1998. Enhancement of exopolysaccharde production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 with a simplified defined medium. Appl. Environ. Microbiol. **64:** 1333-1337.

Looijesteijn, P.J. and Hugenholtz, J. 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its synthesis. J. Biosci. Bioeng. **88:** 178-182. (this thesis chapter 2)

Looijesteijn, P.J., Boels, I.C., Kleerebezem, M. and Hugenholtz, J. 1999. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. **65:** 5003-5008. (this thesis chapter 4)

Marshall, V.M., Cowie, E.N. and Moreton, R.S. 1995. Analysis and production of two exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* LC330. J. Dairy Res. **62:** 621-628.

Mengistu, Y., Edward, C. and Saunders, J.R. 1994. Continuous culture studies on the synthesis of capsular polysaccharide by *Klebsiella pneumoniae* K1. J. Appl. Bacteriol. **76:** 424-430.

Otto, R. 1984. Uncoupling of growth and acid production in *Streptococcus cremoris*. Arch. Microbiol. **140:** 225-230.

Petit, C., Grill, J.P., Maazouzi, N. and Marczak, R. 1991. Regulation of polysaccharide formation by *Streptococcus thermophilus* in batch and fed-batch cultures. Appl. Microbiol. Biotechnol. **36:** 216-221.

Poolman, B. and Konings, W.N. 1988. Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. J. Bacteriol. **70:** 700-707.

Ruijssenaars, H.J., Stingele, F. and Hartmans, S. 2000. Biodegradability of food-associated extracellular polysaccharides. Curr. Microbiol. **40:** 194-199.

Sutherland, I.W. 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23:** 79-150.

Sutherland, I.W. 1994. Structure-function relationships in microbial exopolysaccharides. Biotechnol. Adv. **12:** 393-448.

Tempest, D.W. 1978. The Biochemical significance of microbial growth yields: a reassessment. Tibs **3:** 180-184.

Tuinier, R., Zoon, P., Olieman, C., Cohen Stuart, M.A., Fleer, G.J. and de Kruif, C.G. 1999a. Isolation and physical characterization of an exocellular polysaccharide. Biopolymers **49:** 1-9.

Tuinier, R., Zoon, P., Cohen Stuart, M.A., Fleer, G.J. and de Kruif, C.G. 1999b. Concentration and shear-rate dependence of an exocellular polysaccharide. Biopolymers **50:** 641-646.

van den Berg, D.J.C., Robijn, G.W., Janssen, A.C., Giuseppin, M.L.F., Vreeker, R., Kamerling, J.P., Vliegenthart, J.F.G., Ledeboer, A.M. and Verrips, C.T. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. Appl. Environ. Microbiol. **61:** 2840-2844.

van Casteren, W.H.M., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1998. Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40. Carbohydr. Polym. **37:** 123-130.

van Casteren, W.H.M., Kabel, M.A., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1999. Endoglucanase V and a phosphatase from *Trichoderma viri*de are able to act on modified exopolysaccharide from *Lactococcus lac*tis subsp. *cremoris* B40. Carbohydr. Res. **317:** 131-144.

van Casteren, W.H.M., de Waard, P., Dijkema, C., Schols, H.A. and Voragen, A.G.J. 2000. Structural characterisation and enzymatic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B891. Carbohydr. Res.: in press.

van Kranenburg, R., Marugg J.D., van Swam, I.I., Willem, N.J. and de Vos, W.M. 1997. Molecular characterization of the plasmid-encode eps gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:** 387-397.

van Kranenburg, R., Vos, H.R., van Swam, I.I., Kleerebezem, M., and de Vos, W.M. 1999. Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other gram-positive cocci: complementation, expression and diversity. J. Bacteriol. **181:** 6347-6353.

Chapter 4

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. *Lactobacillus 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 *Streptococcus salivarius* subsp. *thermophilus* are also significantly influenced by the carbon source (Cerning *et al.*, 1994; Gancel and Novel, 1994; Gamar *et al.*, 1997). In chapter two report we described that *Lactococcus 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 *Lactococcus 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 *Lactococcus lactis* subsp. *cremoris*.

MATERIALS AND METHOD

Bacterial strains, culture conditions and analysis of growth and product formation

Bacterial strains and plasmids used in this study are listed in Table 4.1. Fermentations with *Lactococcus 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 ß-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 $litre^{-1}$. *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 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 pyrophosphorylase, and dTDP-glucose pyrophosphorylase cell debris was not removed because these enzymes are probably linked to the cell membranes.

Table 4.1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Reference
Strains		
Lactococcus 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		Cassadaban and Cohen, 1980
Plasmids		
pNZ4000	40-kb plasmid encoding EPS production	van Kranenburg *et al.*, 1997
pNZ4030	Em^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

Lac^+, lactose-fermenting phenotype; Eps^+, exopolysaccharide-producing phenotype; Rf^R, rifampicin resistant; Sm^R, streptomycin resistant; Em^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 cell free extract. 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 cell free extract. 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 cell free extract. 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 cell free extract. 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 cell free extract. 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 cell free extract. The reaction was started by adding 5 mM fructose-1,6-diphosphate (Grobben *et al.*, 1996).

Sugar nucleotide analysis

Cell free extracts were prepared as described above. Immediately after preparation of the cell free extracts, 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

Lactococcus lactis MG1614 harbouring plasmid pNZ4040 and MG1614 harbouring both pNZ4040 and the EPS plasmid pNZ4030 were used to determine the activity of the promoter of the *eps* operon (Table 4.1). Plasmid pNZ4040 contains the *eps* promoter fused to the promoterless *gus*A reporter gene, which encodes ß-glucuronidase (van Kranenburg *et al.*, 1997). The activity of ß-glucuronidase was determined in an assay with 950 μl of GUS buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM ß-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100) and 40 μl of cell free extract. The reaction was started by adding 10 μl of 100 mM *para*-nitro-ß-D-phenyl-glucuronic acid. The increase in the A_{405} was measured at 37°C (Platteeuw *et al.*, 1994). The ß-glucuronidase activity was measured with cell free extracts of MG1614 harbouring pNZ4040 or of MG1614 harbouring pNZ4040 and pNZ4030 to make it possible to distinguish between regulation by chromosomally or plasmid encoded factors.

Controlled overexpression of the *fbp* gene

The *Escherichia 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'-CATGCCATGGCAAAAACGTTAGGTGAATTTATTGTCG-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- and *Xba*I-digested PCR product in *Nco*I-*Xba*I digested pNZ8048, yielding pNZ4150. Plasmid pNZ4150 was transformed into *Lactococcus 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 *Lactococcus lactis* NZ9000 harbouring pNZ4150 by means of electroporation.

Lactococcus 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 A (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

In chapter two we showed that the natural-EPS-producing strain *Lactococcus 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 4.2).

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 (not shown).

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 *gusA* reporter gene, which encodes ß-glucuronidase, under the control of the *eps* promoter. The activity of the *eps* promoter was determined by measuring the ß-glucuronidase activity with cell free extracts of strain MG1614 harbouring pNZ4040 (and pNZ4030) grown in CDM with either glucose or fructose as the source of sugar. The activity of ß-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.

Table 4.2

EPS production by *Lactococcus 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})	UDP-glucose (µmol g protein^{-1})	UDP-galactose (µmol g protein^{-1})
NIZO B40	Glucose	460	29.9 ± 6.4[a]	9.6 ± 2.2[a]
	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 the concentration of sugar nucleotides

Lactococcus 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. 4.1). 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 4.2). The amounts of sugar nucleotides present in cells grown on glucose were higher for strain MG1614 than for strain NZ4010 (Table 4.2). 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 (not shown).

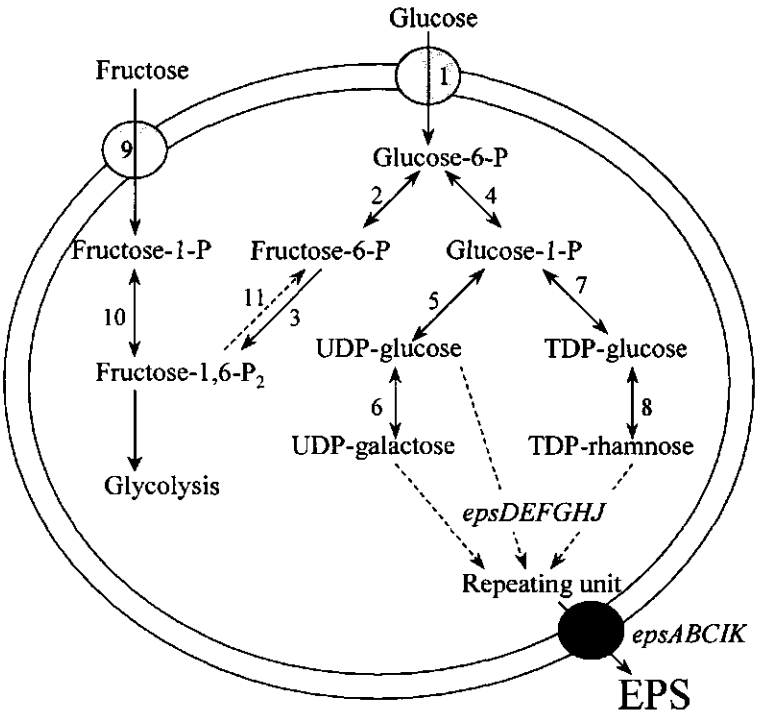

Figure 4.1

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-Phosphofructokinase, 4: α-Phosphoglucomutase, 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-Phosphofructokinase, 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).

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 4.3). 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. 4.1.), it is presumably induced by the presence of fructose. All the other enzymes were not significantly influenced by the source of sugar (Table 4.3).

Striking is the fact that the activity of FBPase was considerably lower than the activities of other enzymes involved in precursor formation and seemed to be even somewhat lower in fructose grown cultures (Table 4.3). 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. 4.1).

Table 4.3

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 *Lactococcus lactis* NZ4010 and MG1614.

Enzyme	Glucose		Fructose	
	NZ4010	MG1614	NZ4010	MG1614
Mannose PTS	29 ± 13[a]	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

Overexpression of *fbp*

To verify if the low activity of FBPase is the bottleneck for 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 A in the medium resulted in transcription of the *fbp* gene. An SDS-polyacrylamide gel of cell free extracts of strain NZ9000 harbouring pNZ4150 showed the appearance of a protein band when the bacteria were induced with nisin (not shown). This protein band had a molecular mass of 36.8 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 4.4

Concentration of EPS and activity of FBPase of strain NZ9000 carrying pNZ4030 and pNZ4015 or pNZ8048 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	nisin (ng ml^{-1})	EPS (mg l^{-1})	OD_{600}	EPS (mg OD^{-1})	μ^* (h^{-1})	FBPase (nmol mg protein^{-1} min^{-1})
NZ9000+pNZ4030+pNZ8048	0	1.4	1.045	1.37	0.22	6.2
NZ9000+pNZ4030+pNZ8048	1	1.6	1.115	1.37	0.27	6.3
NZ9000+pNZ4030+pNZ4150	0	1.3	1.07	1.21	0.23	6.7
NZ9000+pNZ4030+ pNZ4150	0.001	1.5	1.12	1.34	0.27	11.4
NZ9000+pNZ4030+ pNZ4150	0.01	2.4	1.305	1.84	0.47	78
NZ9000+pNZ4030+ pNZ4150	0.1	5.4	1.63	3.31	0.60	303
NZ9000+pNZ4030+ pNZ4150	1	8.5	1.72	4.94	0.74	628

* 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 harbouring 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 harbouring pNZ4030 and the control plasmid pNZ8048, a fivefold increase in EPS production per ml was obtained with an induction level of 1 ng of nisin ml^{-1} (Table 4.4). 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 4.4). Apparently, the activity of FBPase was not only limiting for EPS production but also limiting 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 4.4). To exclude the influence of the growth rate on EPS production, strain NZ9000 harbouring 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, respectively, the uninduced and the induced cultures.

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 4.5). 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 4.5

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

Strain	sugar source	mol% rhamnose	mol% galactose	mol% 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 *Lactococcus lactis* subsp. *cremoris* 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 synthesise EPS (Sutherland, 1972). For *Lactococcus 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 *Lactococcus 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 *Lactobacillus 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 *Lactococcus 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 4.3), 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 *Lactobacillus*

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 found indeed 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 (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 *Lactococcus lactis* subsp. *cremoris* 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 *Lactococcus 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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REFERENCES

Ashtaputre, A.A. and Shah, A.K. 1995. Studies on the exopolysaccharide from *Shingomonas paucimobilis*-GS1: nutritional requirement and precursor-forming enzymes. Curr. Microbiol. **31:** 234-238.

Benthin, S., Nielsen, J. and Villadsen, J. 1993a. Transport of sugars via two anomer-specific sites on mannose-phosphotransferase system in *Lactococcus cremoris*: in vivo study of mechanism, kinetics, and adaptation. Biotechnol. Bioeng. **42:** 440-448.

Benthin, S., Nielsen, J. and Villadsen, J. 1993b. Two uptake systems for fructose in *Lactococcus lactis* subsp. *cremoris* FD1 produce glycolytic and gluconeogenic fructose phosphates and induce oscillations in growth and lactic acid formation. Appl. Environ. Microbiol. **59:** 3206-3211.

Bernstein, R.L. 1965. Control aspects of uridine 5'-diphosphate glucose and thymidine 5'-diphosphate glucose synthesis by microbial enzymes. J. Biol. Chem. **240:** 391-397.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

Cassadaban, M.J. and Cohen, S.N. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:** 179-207.

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87:** 113-130.

Cerning, J., Renard, C.M.G.C., Thibault, J.F., Bouillanne, C., Landon, M., Desmazeaud, M. and Topisirovic, L. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. Appl. Environ. Microbiol. **60:** 3914-3919.

de Ruyter, P.G.G.A., Kuipers, O.P. and de Vos, W.M. 1996. Controlled gene expression systems for *Lactococcus lactis* using the food-grade inducer nisin. Appl. Environ. Microbiol. **62:** 3662-3667.

Gamar, L., Blondeau, K. and Simonet, J.–M. 1997. Physiological approach to extracellular polysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. **83:** 281-287.

Gancel, F. and Novel, G. 1994. Exopolysaccharide production by *Streptococcus salivarius* ssp. *thermophilus* cultures. 1. Conditions of production. J. Dairy Sci. **77:** 685-688.

Gasson, M.J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:** 1-9.

Gopal, P.K. and Reilly, K.I. 1995. Molecular architecture of the lactococcal cell surface as it relates to important industrial properties. Int. Dairy J. **5:** 1095-1111.

Grant, W.D., Sutherland, I.W. and Wilkinson, J.F. 1970. Control of colanic acid synthesis. J. Bacteriol. **103:** 89-96.

Grobben, G.J., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1996. Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. Appl. Microbiol. Biotechnol. **46:** 279-284.

Grobben, G.J., van Casteren, W.H.M., Schols, H.A., Oosterveld, A., Sala, G., Smith, M.R., Sikkema, J. and de Bont, J.A.M. 1997. Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and on fructose. Appl. Microbiol. Biotechnol. **48:** 516-521.

Hamilton, W.D.O., Harrison, D.A. and Dyer, T.A. 1988. Sequence of the *Escherichia coli* fructose-1,6-bisphosphatase gene. Nucleic Acids Res. **16:** 8707.

Harding, N.E., Raffo, S., Raimondi, A., Cleary, J.M. and Ielpi, L. 1993. Identification, genetic and biochemical analysis of genes involved in synthesis of sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. **139:** 447-457.

Horan, N.J., Jarman, T.R. and Dawes, E.A. 1981. Effects of carbon source and inorganic phosphate concentration on the production of alginic acid by a mutant of *Azotobacter vinelandii* and on the enzymes involved in its biosynthesis. J. Gen. Microbiol. **127:** 185-191.

Kuipers, O.P., de Ruyter, P.G.G.A., Kleerebezem, M. and de Vos, W.M. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:** 15-21.

Leitão, J.H. and Sá-Correia, I. 1995. Growth-phase-dependent alginate synthesis, activity of biosynthetic enzymes and transcription of alginate genes in *Pseudomonas aeruginosa*. Arch. Microbiol. **163:** 217-222.

Looijesteijn, P.J. and Hugenholtz, J. 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its exopolysaccharide synthesis. J. Biosci. Bioeng. **88:** 178-182. (this thesis chapter 2)

Norval, M. and Sutherland, I.W. 1973. The production of enzymes involved in exopolysaccharide synthesis in *Klebsiella aerogenes* types 1 and 8. Eur. J. Biochem. **35:** 209-215.

Otto, R. 1984. Uncoupling of growth and acid production in *Streptococcus cremoris*. Arch. Microbiol. **140:** 225-230.

Petit, C., Grill, J.P., Maazouzi, N. and Marczak, R. 1991. Regulation of polysaccharide formation by *Streptococcus thermophilus* in batch and fed-batch cultures. Appl. Microbiol. Biotechnol. **36:** 216-221.

Platteeuw, C., Simons, G. and de Vos, W.M. 1994. Use of *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60:** 587-593.

Qian, N., Stanley, G.A., Hahn-Hägerdal, B. and Rådström, P. 1994. Purification and characterization of two phosphoglucomutases from *Lactococcus lactis* subsp. *lactis* and their regulation in maltose- and glucose-utilizing cells. J. Bacteriol. **176:** 5304-5311.

Sikkema, J. and Oba, T. 1998. Extracellular polysaccharides of lactic acid bacteria. Snow Brand R&D Rep. **107:** 1-31.

Sutherland, I.W. 1972. Bacterial exopolysaccharides. Adv. Microb. Physiol. **8:** 143-213.

Sutherland, I.W. 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23:** 79-150.

van Casteren, W.H.M., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1998. Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40. Carbohydr. Polym. **37:** 123-130.

van den Berg, D.J.C., Robijn, G.W., Janssen, A.C., Giuseppin, M.L.F., Vreeker, R., Kamerling, J.P., Vliegenthart, J.F.G., Ledeboer, A.M. and Verrips, C.T. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. Appl. Environ. Microbiol. **61:** 2840-2844.

van Kranenburg, R., Marugg, J.D., van Swam, I.I., Willem, J. and de Vos, W.M. 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:** 387-397.

van Kranenburg, R., van Swam, I.I., Marugg, J.D., Kleerebezem, M. and de Vos, W.M. 1999. Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. J. Bacteriol. **181:** 338-340.

Van Riel, J. and Olieman, C. 1991. Selectivity control in the anion-exchange chromatographic determination of saccharides in dairy products using pulsed amperometric detection. Carbohydr. Res. **215:** 39-46.

Williams, A.G. and Wimpenny, J.W.T. 1980. Extracellular polysaccharide biosynthesis by *Pseudomonas* NCIB 11264. Studies on precursor-forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J. Gen. Microbiol. **116:** 133-141.

Chapter 5

Physiological function of exopolysaccharides produced by *Lactococcus lactis*

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ABSTRACT

The physiological function of EPS produced by *Lactococcus lactis* was studied by comparing the tolerance of the non-EPS-producing strain *Lactococcus lactis* subsp. *cremoris* MG1614 and an EPS-producing isogenic variant of this strain to several anti-microbial factors. There was no difference in the sensitivity of the strains to increased temperatures, freezing or freeze-drying and the antibiotics, penicillin and vancomycin. A model system showed that EPS production did not affect the survival of *Lactococcus lactis* during passage through the gastrointestinal tract although the EPS itself was not degraded during this passage. The presence of cell associated EPS and EPS in suspension resulted in an increased tolerance to copper and nisin. Furthermore, cell associated EPS also protected the bacteria against bacteriophages and the cell wall degrading enzyme lysozyme. However, it has not been possible, so far, to increase EPS-production using the presence of copper, nisin, lysozyme or bacteriophages as inducing factors.

INTRODUCTION

Several lactic acid bacteria as well as numerous other micro-organisms produce extracellular polysaccharides (EPSs). EPSs either surround the bacterial cells as a capsule or are excreted into the extracellular environment as slime, although the distinction between these two types of EPSs is not always very clear (Whitfield, 1988).

In the last decade, most studies on EPS produced by lactic acid bacteria have focussed on the influence of physiological growth conditions on the EPS biosynthesis, genetics of EPS biosynthesis, and elucidation of the composition and primary structures of these EPSs. Information about the physiological role of EPSs for the producing lactic acid bacteria themselves is almost completely lacking. Apparently, EPSs have some kind of biological function, because it is very unlikely that bacteria use both substrate and energy for the production of useless metabolites (Dudman, 1977). Some bacteria invest more than 70% of their energy in EPS production, presumably to obtain a selective advantage in the environment (Weiner *et al.*, 1995). In addition EPS synthesis is a relatively stable property and EPS-producing organisms have a stable presence in various environments. EPSs are obviously not essential for bacteria because enzymatic or physical removal of EPS does not negatively affect cell growth *in vitro* and mutants unable to produce EPS appear spontaneously (Schellhaass, 1983). Most proposed functions of EPSs in general are of a protective nature such as protection against dehydration, macrophages, bacteriophages, protozoa, antibiotics and toxic compounds (Whitfield, 1988; Weiner *et al.*, 1995; Roberts, 1996). Other possible functions of EPS include sequestering of essential cations (Weiner *et al.*, 1995) and involvement in adhesion and biofilm formation (Roberts, 1996).

The aim of this study is to obtain more insight into the physiological function of EPS produced by *Lactococcus lactis* subsp. *cremoris* by comparing the sensitivity of the EPS-producing strain NZ4010 and the non-producing isogenic parent strain MG1614 to various anti-microbial factors. The genes necessary for EPS production by strain NZ4010 are encoded on a plasmid and the EPS produced by this strain is composed of glucose, rhamnose, galactose and phosphate in a ratio of 2:2:1:1 (Fig. 5.1a; van Kranenburg *et al.*, 1997). Most of the EPS that is produced by *Lactococcus lactis* NZ4010 is excreted into the environment but some of the EPS is attached to the bacteria. The thickness of the layer of this cell-associated EPS depends on the culture conditions that are applied (Looijesteijn and Hugenholtz, 1999). Fig. 5.1b shows cells of strain NZ4010 with a thick layer of cell-associated EPS. Furthermore, to make a distinction between the protection by cell-associated EPS and by the presence of EPS in the medium, we used cultures of MG1614 supplemented with EPS, as a control. We will refer to these cultures as cultures with EPS in suspension. Knowledge of the biological role of EPS might lead to improvement of the relatively low production yields or to insight into the regulation mechanisms that are involved in EPS biosynthesis by *Lactococcus lactis*.

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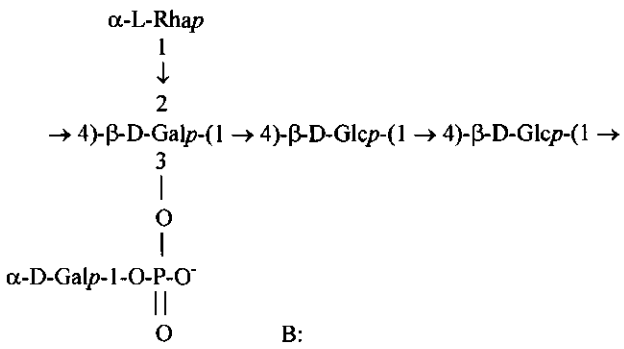

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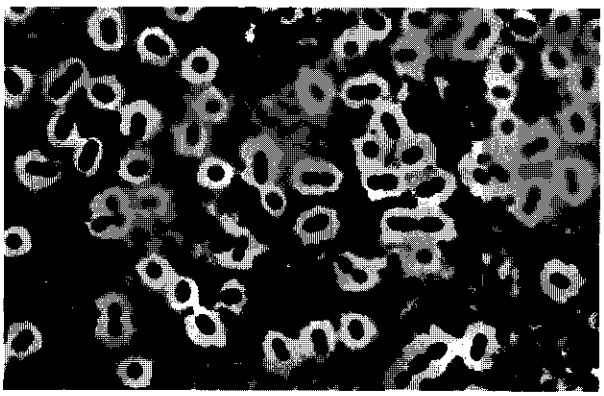

Figure 5.1

A: Primary structure of the repeating unit of EPS produced by *Lactococcus lactis* subsp. *cremoris* NZ4010 (van Kranenburg *et al.* 1997). B: Cells of *Lactococcus lactis* subsp. *cremoris* NZ4010 with a layer of cell associated EPS.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and EPS

Stock cultures of *Lactococcus lactis* subsp. *cremoris* strain NZ4010 (van Kranenburg *et al.*, 1997) and strain MG1614 (Gasson, 1983) were kept in litmus milk with 1% glucose (-40°C). *Lactococcus lactis* MG1614 harbouring pNZ4030 (van Kranenburg *et al.*, 1997; Looijesteijn *et al.*, 1999) was kept in GM17 with 10% glycerol and 5 mg l^{-1} erythromycin at –40°C. Phage ML3 that is lytic for *Lactococcus lactis* MG1614 was kept at –40°C in GM17 with 10% glycerol.

EPS, added in several experiments as a control, was produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 (van Kranenburg *et al.*, 1997; Looijesteijn and Hugenholtz, 1999) and isolated as described previously (Tuinier *et al.*, 1999). EPSs produced by strain NIZO B40 and NZ4010 have identical chemical structures (van Kranenburg *et al.*, 1997).

Analysis of bacterial growth, EPS concentration and phage titre

Cell growth was determined by measuring the optical density of the culture fluid at 600 nm (OD_{600}) or by determination of the number of colony forming units (cfu) on tryptone glucose meat extract agar (TGMA) after an incubation of 2 days at 30°C. TGMA contains 1% tryptone, 0.3% meat extract, 0.5% yeast extract, 2% glucose, 4% tomato juice, 0.1% Tween 80, 0.2% K_2HPO_4 and 1.5% agar (pH 6.9).

The amount of EPS was measured by gelpermeation chromatography using dextran as standard as described previously (Looijesteijn and Hugenholtz, 1999).

In order to determine the number of plaque forming units of phage ML3, appropriate dilutions of phage containing culture fluid were mixed with GM17-soft agar, $CaCl_2$ and the different *Lactococcus lactis* strains and poured on a GM17 agar plate containing 10 mM $CaCl_2$. The number of plaques and the diameter of the plaques were determined after an incubation of 48 hours at 30°C.

Exposure to several anti-microbial factors

To compare the sensitivity of strain NZ4010 and MG1614 to several anti-microbial compounds and conditions four types of experiments were used:

Exposure of harvested cells to a high concentration of the anti-microbial substance

Lactococcus lactis subsp. *cremoris* NZ4010 and MG1614 were grown in a fermenter at 30°C and pH 6.3. The formation of lactic acid was neutralised by automatic titration with NH_4OH, which resulted in a maximal layer of EPS around the cells of strain NZ4010 (Looijesteijn and Hugenholtz, 1999). The bacteria were grown until an OD_{600} of about 3 was reached and harvested by centrifugation (30 min, 16,000 g, 4°C). The cell pellet was 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) and kept at -40°C.
The harvested bacteria were suspended in GM17 medium to which an anti-microbial compound was added with a starting concentration of about 5×10^8 cfu ml^{-1} and incubated at 30°C, or 37°C for incubations with lysozyme.

Exposure of growing cultures to anti-microbial conditions

Overnight precultures (1%) in GM17 at 30°C were used to start the experiments. *Lactococcus lactis* subsp. *cremoris* NZ4010 and MG1614 were grown in GM17 at 30°C until a certain OD_{600} was reached. At that moment, the anti-microbial component was added to the cultures or the culture fluids with or without exogenously added EPS (1 g l^{-1}) were frozen (-40°C), freeze-dried or exposed to increased temperatures (40, 50 and 60 and 70°C). The OD and the number of cfu were determined with time.

Bioassays

One hundred µl of an overnight culture was mixed with 15 ml of TGMA in an agar plate. After drying, 7 holes of 3.5 mm were made in each plate. The holes were filled with 20 µl of different concentrations of the anti-microbial compounds. The diameter of the clear zone was determined after an incubation of 2 days at 30°C.

pH-controlled fermentations

Fermentations were performed in bottles filled with 500 ml of M17 supplemented with 6% glucose and different concentrations of anti-microbial compounds at 30°C. An inoculum (1%) of an overnight culture at 30°C was used to start the fermentations. The pH was kept at 5.8 by automatic titration with 5 M NaOH. Nitrogen gas was led over the culture medium. Fermentations were ended when titration of NaOH had stopped.

Passage through the gastrointestinal canal

The human gastrointestinal tract was simulated by suspension of harvested cells of strain MG1614 and NZ4010 in GM17 (pH 4.0) containing 5 µg ml^{-1} of pepsin and incubated for 90 min at 37°C. The pH was increased to 6.5 with NaOH. Corolase PP (Rhöm, Germany), which is a mixture of pancreatic enzymes, cholic acid and taurodeoxy cholic acid were added at a concentration of respectively 10 µg ml^{-1}, 0.5 mM and 10 mM and the incubation was continued for 150 min at 37°C (Alting *et al.*, 1997).

The resistance of EPS during passage through the gastrointestinal tract was tested *in vivo*. During an experimental period of two weeks, six male 8-9 weeks old Wistar rats were fed with an EPS-containing diet. The rats were housed individually in metabolic cages. The pasteurised diet consisted of lactase treated milk fermented by *Lactococcus lactis* NIZO B40 that was supplemented with extra EPS, rice flour, cellulose, vitamins and Fe-citrate according to the standard diet AIN-93 for laboratory rodents (Reeves *et al.*, 1993). Food intake and body weight were recorded and urine and faeces were collected during the experimental period. Faeces were freeze-dried for dry weight determination. Faecal water was prepared by reconstituting freeze-dried samples with demineralised water to 30% dry weight. After homogenisation, the samples were centrifuged and the EPS concentration in the supernatant was determined.

RESULTS

Protection against bacteriophages

The EPS-producing strain *Lactococcus lactis* NZ4010 was less sensitive to bacteriophages than the non-producing strain MG1614 (Table 5.1). Apparently the EPS layer around the EPS-producing bacteria is responsible for the reduction of the phage sensitivity because

addition of EPS did not reduce the sensitivity of strain MG1614 for bacteriophages. To make sure that EPS layer was responsible for the reduction in phage sensitivity and not other phage protection mechanisms that could possibly be encoded on the 42-kb *eps* plasmid pNZ4000 from NZ4010, strain MG1614 harbouring pNZ4030 was used as a control. Next to the replicon, plasmid pNZ4030 only encodes EPS production, a putative cobalt transport system, and erythromycin resistance (van Kranenburg *et al.*, 1997; 2000). The sensitivity to bacteriophages of this strain and strain NZ4010 were the same. Besides a reduction of the phage titre, a decrease of the plaque diameter was observed for the EPS-producing strains (Table 5.1).

Table 5.1

Number of plaque forming units per ml and plaque diameter found with various *Lactococcus lactis* subsp. *cremoris* strains using a suspension of phage ML3.

Strain	number of plaques ml^{-1}	Plaque diameter (mm) ($n \geq 16$)
MG1614	$8.4 * 10^8$	2.61 ± 0.41
NZ4010	$4.1 * 10^8$	1.54 ± 0.12
MG1614+pNZ4030	$4.3 * 10^8$	1.47 ± 0.18
MG1614+EPS	$1.0 * 10^9$	2.47 ± 0.34

Compared with strain MG1614, the reduction of the sensitivity of strain NZ4010 to phages was about 50%. This relatively low decrease of the sensitivity has a major impact on the development of the cultures when infected with a low concentration of phages (Fig. 5.2).

Protection against increased and decreased temperatures

EPS was not able to protect the bacteria against increased temperatures. The decrease of the number of viable cells with time, when growing bacteria of strain MG1614, NZ4010 or MG1614 with EPS in suspension were exposed to temperatures of 40, 50, 60 and 70°C, was comparable for all three cultures at each temperature. EPS did not protect the bacteria against low temperatures either. Over a period of 162 days cultures of the three strains, which were kept at -40°C, were repeatedly subjected to thawing at 4°C and freezing at -40°C. After 25 such cycles, the number of viable cells was decreased to 1-4% of the initial number of viable bacteria for all three cultures. Although EPS provides the EPS-producing bacteria with a hydrophilic layer, no difference in the survival of the bacteria was found when the cultures were freeze-dried either. Survival after freeze-drying of the bacteria of strain NZ4010, MG1614 and MG1614 with 1 g l^{-1} EPS in suspension, was 30.3%, 32.2% and 32.1%, respectively.

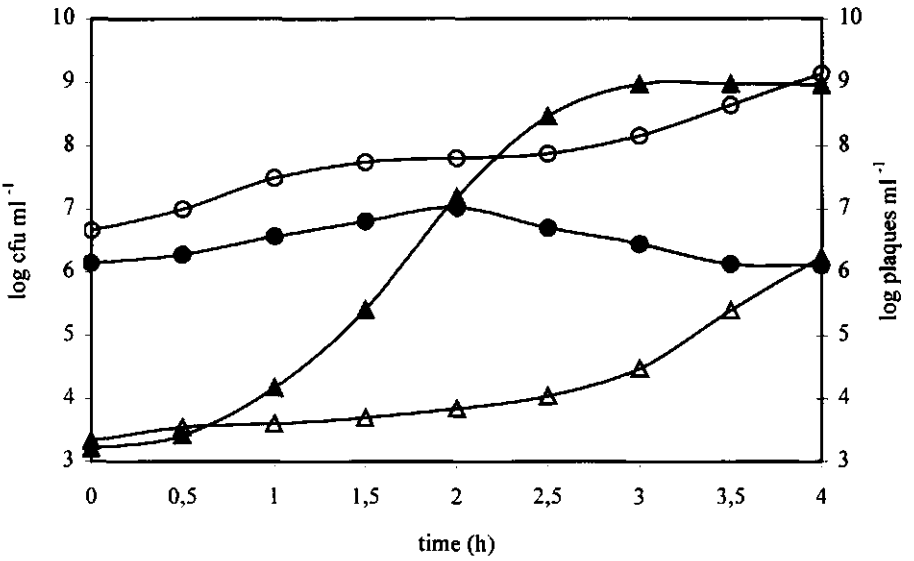

Figure 5.2

The number of viable bacteria of *Lactococcus lactis* MG1614 (●) and MG1614+pNZ4010 (○) and the concentration of phage ML3 in these cultures (▲,△) during an incubation in M17 containing 2% of glucose and 10 mM of $CaCl_2$ at 30°C.

Influence of EPS on the tolerance of *Lactococcus lactis* to metal ions

Table 5.2

Influence of copper on the production of EPS by *Lactococcus lactis* subsp. *cremoris* NZ4010.

$[Cu^{2+}]$ (mM)	EPS production ($mg\ l^{-1}$)
0	400
0.3	290
0.8	200
1.1	10
1.4	<10*

* Microscopy analysis revealed the presence of a thin layer of cell-associated EPS under these culture conditions but this amount of EPS was too low for an exact determination.

The possible protection of EPS against copper was investigated using pH-controlled fermentations. The presence of copper ions during these fermentations negatively influenced the growth of *Lactococcus lactis*: the growth rate decreased, the lag phase increased and the

final optical density decreased with increasing copper concentration. The presence of EPS partially abolished the effect of copper on growth of *Lactococcus lactis*. Strain MG1614 was not able to grow at pH 5.8 and 30°C in medium with a concentration of 1.4 mM of $CuSO_4$ whereas the EPS-producing parent strain could still grow at this concentration of copper. Addition of EPS to cultures of MG1614 improved the growth of this organism at elevated copper concentrations (Fig. 5.3).

The presence of Cu^{2+} could not be used as a tool to increase the EPS production by strain NZ4010. The EPS concentration in the medium decreased with increasing concentrations of copper (Table 5.2).

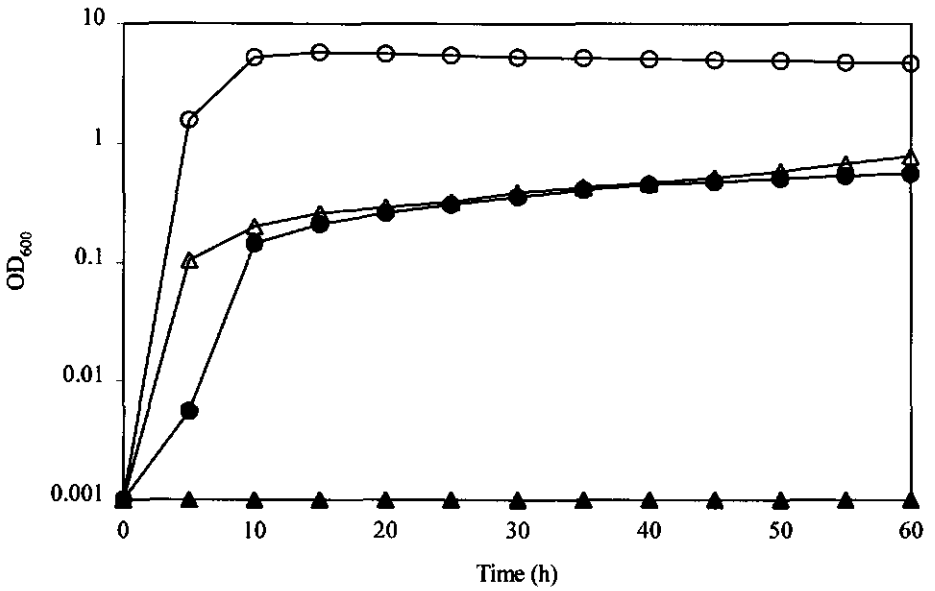

Figure 5.3

Growth of MG1614 without Cu^{2+} (○), NZ4010 with 1.4 mM Cu^{2+} (●), MG1614 with 1.4 mM Cu^{2+} (▲) and MG1614 with 1.4 mM Cu^{2+} and EPS (△) in M17 with 6% of glucose, pH 5.8 and 30°C.

Protection against lysozyme and antibiotics

Lysozyme is an enzyme that disrupts the cell walls of certain bacteria by hydrolysing the glycosidic β(1→4) bond between N-acetyl-D-muramic acid and N-acetyl-D-glucosamine of peptidoglycan, resulting in lysis of the bacteria. Harvested cells of strain NZ4010 and MG1614 with or without EPS were suspended in GM17 with 1 g l^{-1} of lysozyme (50,000 U mg^{-1}) and incubated at 37°C, which is the optimal hydrolysing temperature for this enzyme. A thick layer of EPS (Fig. 5.1b) surrounded the cells of strain NZ4010. These cells were less susceptible to lysozyme than the cells of the non-EPS-producing strain MG1614. Apparently,

cell-associated EPS was responsible for the protection against lysozyme because addition of EPS to cultures of MG1614 did not result in increased tolerance of these bacteria (Table 5.3).

Table 5.3

Percentage of survival of *Lactococcus lactis subsp. cremoris* NZ4010 and MG1614 with or without added EPS after exposure to 1g l^{-1} of lysozyme at 37°C.

Incubation time	*Lactococcus lactis subsp. cremoris*		
(hour)	NZ4010	MG1614	MG1614 with EPS
0	100%	100%	100%
1	44.7	22.7	22.7
2	12.6	3.8	3.2
2.5	4.2	1.1	1.1
3	2.0	0.9	0.5

Another possible function of EPS is the protection against antibiotics. The sensitivities of strain NZ4010 and MG1614 to two antibiotics interfering with cell wall biosynthesis, penicillin G and vancomycin, and for the lantibiotic nisin were compared. Penicillin G inhibits bacterial cell wall synthesis during active multiplication by preventing cross-linking of peptidoglycan strands. Vancomycin kills bacteria by blocking the cell wall biosynthesis through binding tightly to the D-alanyl-D-alanine portion of cell wall precursor. One possible mechanism for the protection of EPS against antibiotics is by preventing the passage of the active compounds to the cells. This could be especially important for antibiotics with a high molecular mass such as vancomycin.

In contrast to the experiments with lysozyme, the rate of mortality was very low when harvested cells of MG1614 or NZ4010 were exposed to concentrations of penicillin or vancomycin that were far above the MIC. The bacteria are harvested at the end of the exponential phase and the antibiotics are only active during multiplication of the bacteria, which could explain this observation. The addition of penicillin or vancomycin to actively growing cultures of NZ4010 and MG1614 with or without EPS in suspension at the moment an OD_{600} of about 0.5 was reached did not result in significant differences. In bioassays no difference could be detected in the sensitivity of both strains to penicillin and vancomycin either.

Nisin is a positively charged bacteriocin that is produced by some *Lactococcus lactis* strains. The small anti-microbial peptide is active against a wide range of Gram-positive bacteria amongst which are non-nisin-producing lactococci such as strain MG1614 and NZ4010. Nisin kills growing target bacteria through permeabilisation of the cytoplasmic membrane, resulting in leakage of essential cell components and dissipation of the membrane potential (van Kraaij *et al.*, 1998). Growth in GM17 with different concentrations of nisin was

comparable for strain MG1614 and NZ4010 although this resulted in sedimentation of the cells of strain NZ4010. Addition of 130 ng nisin ml^{-1} to growing cultures when an OD_{600} of 0.5 was reached, resulted in dying off of strain MG1614 but when EPS was added too, this strain continued to grow (Fig. 5.4).

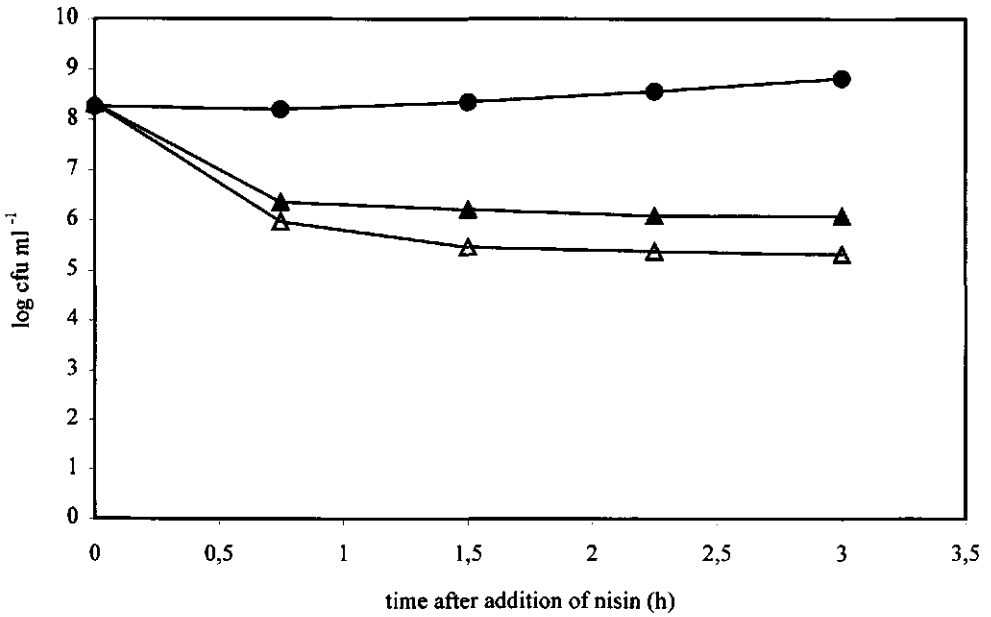

Figure 5.4

Number of viable bacteria after 130 ng nisin ml^{-1} was added to growing cultures of *Lactococcus lactis* NZ4010 (▲), MG1614 (△) and MG1614 with EPS (●) at the moment that an OD_{600} of 0.5 was reached.

Effect of EPS on survival of gastrointestinal tract passage

The possible influence of EPS on the survival of the bacteria during passage through the gastrointestinal tract was tested using a model system simulating the process of digestion in humans. The presence of EPS did not exert any protective effect for *Lactococcus lactis* during the passage through the intestine, only 0.1% of both strain MG1614 and NZ4010 survived this passage.

It is obvious that EPSs can only exert a positive influence on the passage of bacteria through the intestine when the EPSs itself can withstand digestion. The resistance of EPS against digestion was tested *in vivo* using rats. The rats were fed with a porridge that contained 346 mg EPS kg^{-1}. On average, they produced 4.44 g of faeces (dry weight) during a period of 4 days when administered 296 g of porridge, which contained in total 102 mg of EPS. The amount of EPS found in the faeces was 22.2 mg g^{-1} (98 mg of EPS per 4.44 g of

faeces). Hence, the total recovery of EPS was 96%. The retention times found with gel permeation chromatography, which is a measure for the molecular mass of polysaccharides, for EPS before and after digestion were similar. Apparently, *in vivo* EPS is not broken down during passage through the gastrointestinal tract.

DISCUSSION

EPS produced by *Lactococcus lactis* subsp. *cremoris* NZ4010 cannot be used as an energy source by the producing organism itself (not shown) but it protects the bacteria against several anti-microbial factors such as bacteriophages, metal ions, nisin and lysozyme. For some of these factors the presence of a layer of cell-associated EPS is essential for the protection whereas for other anti-bacterial agents EPS in suspension also protects them.

Lactococcus lactis NZ4010 produces an EPS that is composed of glucose, galactose, rhamnose and phosphate (Fig. 5.1a; van Kranenburg *et al.*, 1997). Due to the presence of phosphate the EPS is negatively charged above a pH of 2 (Tuinier *et al.*, 1999). This negative charge probably is important for the protective function of EPS against the toxic metal ions and the positively charged lantibiotic nisin. Apparently, the detoxifying mechanism of EPS in these cases is scavenging of the positively charged molecules.

Previous observations for different organisms (Dudman, 1977; Mittelman and Geesey, 1985) also indicated that extracellular anionic polysaccharide-producing bacteria are less susceptible to heavy metals than the non-producing variants due to a reduction of the free metal ion concentration at the cell surface. Alginate and gellan-producing strains of *Pseudomonas aeruginosa* and *Sphingomonas paucimobilis*, respectively, were however less tolerant to sublethal concentrations of copper than the EPS-defective variants (Richau *et al.*, 1997). In contrast to these strains, the specific EPS production of strain NZ4010 was not stimulated in the presence of Cu^{2+}.

It could also be possible that essential cations are sequestered by the anionic EPS resulting in an increase of the gradient across the cell membranes (Weiner *et al.*, 1995; Decho, 1990). Besides the *eps* genes, plasmid pNZ4000 that codes for EPS production by *Lactococcus lactis* NZ4010, also contains nucleotide sequences that are homologous to genes involved in cobalt and magnesium transport. Under conditions of Mg^{2+} or Co^{2+} limitation the combined action of these genes and the presence of EPS around the bacteria might enhance the uptake of the cations from the medium (van Kranenburg *et al.*, 2000).

Cell-associated EPS was shown to be beneficial in the protection against lysozyme and bacteriophages. Conjugal transfer of plasmid pSRQ2202, encoding EPS production, also resulted not only in a mucoid phenotype but also in resistance to bacteriophages that were lytic to the parent strains (Vedamutu and Neville, 1986) and the bacteriophage adsorption blocking plasmid pCI658 encodes EPS production in *Lactococcus lactis* HO2 (Forde *et al.*,

1999; Forde and Fitzgerald, 1999). Bacteriophage infection starts with the attachment of the phages to the cell surface at specific recognition sides, which for lactococci are found on cell wall polysaccharides and at the cell membrane (Laux and Süssmuth, 1996). The reduction of phage sensitivity for EPS-producing strains could be caused by an inhibition of this adsorption of phages due to coating of the phage receptors at the cell surface by EPS. It is also possible that phages bind to the EPS because of a resemblance with the phage receptors. This is also shown for certain monosaccharides the addition of which resulted in reduced phage sensitivity (Laux and Süssmuth, 1996; Valyasevi *et al.*, 1990). As EPS in suspension, EPS did not result in reduction of the phage sensitivity of strain MG1614, the first mechanism is most likely involved in the reduction of the phage sensitivity of strain NZ4010.

Another generally accepted role of a hydrated polysaccharide layer around the surface of bacteria is the protection from the harmful effects of desiccation (Roberts, 1996). Dried or frozen stable starter cultures with a high population of viable and uninjured cells are needed for various dairy fermentations. The addition of polysaccharides such as pectin or dextran to cultures of lactic acid bacteria were shown to have a positive influence on their survival (Fajardo-Lira *et al.*, 1997). In our experiments the presence of either cell-associated EPS or EPS in suspension did not increase the survival during freezing and freeze-drying. Comparable results were found for the addition of gelatin, xanthan and maltodextrins, before freeze-drying, to cells of *Lactococcus lactis* (Champagne *et al.*, 1996). Exopolysaccharide-producing strains of yoghurt bacteria also did not show increased resistance to reduction of water activity in milk used to produce yoghurt (Fajardo-Lira *et al.*, 1997). In a previous report we described that reduction of the water activity resulted in a decrease of the specific EPS production (Looijesteijn and Hugenholtz, 1999). The maximum concentration of NaCl still allowing growth was higher for strain MG1614 than for strain NZ4010 (not shown). It could be possible that the layer of cell-associated EPS, as it has a high binding capacity for water, also reduces the water activity around the bacteria.

Several exopolysaccharides produced by lactic acid bacteria are claimed to have positive influences on human health (Sikkema and Oba, 1998). Some of these effects are dependent on the resistance of EPS during passage through the intestine. NZ4010 EPS was found unaltered in the faeces of rats fed with an EPS containing diet. The low biodegradability of this type of EPS was also shown by Ruijssenaars *et al.* (2000). The ability to produce EPS did not, however, increase the survival of *Lactococcus lactis* during passage through the gastrointestinal tract.

It is obvious that EPSs produced by different organisms have different physiological functions. Most functions are related to the natural habitat of the producing organisms. The protection of *Lactococcus lactis* NZ4010 EPS against nisin and bacteriophages could be a competitive advantage in mixed strain dairy starter cultures. Unfortunately, we were unable to increase the EPS production by *Lactococcus lactis* subsp. *cremoris* NZ4010 by exposing the organisms to the anti-microbial factors that are described in this paper.

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REFERENCES

Alting, A.C., Meijer, R.J.G.M. and van Beresteijn, E.C.H. 1997. Incomplete elimination of the ABBOS epitope of bovine serum albumin under simulated gastrointestinal conditions of infants. Diabetes Care **20:** 875-880.

Champagne, C.P., Mondou, F., Raymons, Y. and Roy, D. 1996. Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. Food Res. Int. **29:** 555-562.

Decho, A.W. 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanogr. Mar. Biol. Annu. Rev. **28:** 73-153.

Dudman, W.F. 1977. The role of surface polysaccharides in natural environments. In: *Surface carbohydrates of the prokaryotic cell*, ed. Sutherland, I.W., pp. 357-414, Academic Press, London.

Fajardo-Lira, C., García-Garibay, M., Wacher-Rodarte, C., Farrés, A. and Marshall, V.M. 1997. Influence of water activity on the fermentation of yogurt made with extracellular polysaccharide-producing or non-producing starters. Int. Dairy J. **7:** 279-281.

Forde, A., Daly, C. and Fitzgerald, G.F. 1999. Identification of four phage resistance plasmids from *Lactococcus lactis* subsp. *cremoris* HO2. Appl. Environ. Microbiol. **65:** 1540-1547.

Forde, A. and Fitzgerald, G.F. 1999. Analysis of exopolysaccharide (EPS) production mediated by the bacteriophage adsorption blocking plasmid, pCI658, isolated from *Lactococcus lactis* ssp. *cremoris* HO2. Int. Dairy J. **9:** 465-172.

Gasson, M.J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:** 1-9.

Laux, P. and Süssmuth, R. 1996. Activity reduction of lactococcal phages by monosaccharides, D-glucosamine, D-galactosamine, D-glucuronic acid and anti-microbial agents. Milchwissenschaft **51:** 256-258.

Looijesteijn, P.J. and Hugenholtz, J. 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its synthesis. J. Biosci. Bioeng. **88:** 178-182. (this thesis chapter 2)

Looijesteijn, P.J., Boels, I.C., Kleerebezem, M. and Hugenholtz, J. 1999. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. **65:** 50003-5008. (this thesis chapter 4)

Mittelman, M.W. and Geesey, G.G. 1985. Copper-binding characteristics of exopolymers from a freshwater-sediment bacterium. Appl. Environ. Microbiol. **49:** 846-851.

Petit, C., Grill, J.P., Maazouzi, N. and Marczak, R. 1991. Regulation of polysaccharide formation by *Streptococcus thermophilus* in batch and fed-batch cultures. Appl. Microbiol. Biotechnol. **36:** 216-221.

Reeves, P.G., Nielsen, F.H. and Fahey jr., G.C. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123:** 1939-1951.

Richau, J.A., Choquenet, D., Fialho, A.M., Moreira, L.M. and Sá-Correia, I. 1997. The biosynthesis of the exopolysaccharide gellan results in the decrease of *Sphingomonas paucimobilis* tolerance to copper. Enzyme Microb. Technol. **20:** 510-515.

Roberts, I.S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. **50:** 285-315.

Ruijssenaars, H.J., Stingele, F. and Hartmans, S. 2000. Biodegradability of food-associated extracellular polysaccharides. Curr. Microbiol. **40:** 194-199.

Schellhaass, S.M. 1983. Characterization of exocellular slime produced by bacterial starter cultures used in the manufacture of fermented dairy products. Ph.D. Thesis, University of Minnesota, U.S.A.

Sikkema, J. and Oba, T. 1998. Extracellular polysaccharides of lactic acid bacteria. Snow Brand R&D Rep. **107:** 1-31.

Tuinier, R., Zoon, P., Olieman, C., Cohen Stuart, M.A., Fleer, G.J. and de Kruif, C.G. 1999. Isolation and physical characterization of an exocellular polysaccharide. Biopolymers **49:** 1-9.

Valyasevi, R., Sandine, W.E. and Geller, B.L. 1990. The bacteriophage kh receptor of *Lactococcus lactis* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide. Appl. Environ. Microbiol. **56:** 1882-1889.

van Kraaij, C., Breukink, E., Noordermeer, M.A., Demel, R.A., Siezen, R.J., Kuipers, O.P. and de Kruiff, B. 1998. Pore formation by nisin involves translocation of its C-terminal part across the membrane. Biochemistry **37:** 16033-16040.

van Kranenburg, R., Marugg, J.D., van Swam, I.I., Willem, N.J. and de Vos, W.M. 1997. Molecular characterization of the plasmid-encoded *eps* gene culster essential for exopolysaccharide synthesis in *Lactococcus lactis*. Mol. Microbiol. **24:** 387-397.

van Kranenburg, R., Kleerebezem, M. and de Vos, W.M. 2000. Nucleotide sequence analysis of the actococcal EPS plasmid pNZ4000. Plasmid **43:** 130-136.

Vedamuthu, E.R. and Neville, J.M. 1986. Involvement of a plasmid in production of ropiness(mucoidness) in milk cultures by *Streptococcus cremoris* MS. Appl. Environ. Microbiol. **51:** 677-682.

Weiner, R., Langille, S. and Quintero, E. 1995. Structure, function and immunochemistry of bacterial exopolysaccharides. J. Ind. Microbiol. **15:** 339-346.

Whitfield, C. 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. **34:** 415-420.

Chapter 6

General Discussion

The aim of the work described in this thesis was to optimise the EPS production of *Lactococcus lactis* subsp. *cremoris* and to obtain insight in the regulation of EPS biosynthesis. For that reason the optimal culture condition for EPS production by *Lactococcus lactis* NIZO B40 were determined and it was demonstrated that growth and EPS production in this organism could be uncoupled (Chapter 2). Furthermore, the influence of different substrate limitations on the yield and composition of EPS produced by *Lactococcus lactis* NIZO B40 and NIZO B891 was studied (Chapter 3). An explanation was found for the difference in EPS yields of *Lactococcus lactis* NIZO B40 when cultured in media with either glucose or fructose (Chapter 4). Finally, the physiological function of EPS for the producing lactococci was investigated (Chapter 5).

The role of exopolysaccharides produced by lactic acid bacteria in food

Rheological properties

Several exopolysaccharides such as xanthan and gellan are used to achieve the desired rheological properties of (food) products (Sutherland, 1998). Important characteristics of xanthan are a high viscosity at low concentrations and pseudoplasticity (Morris, 1993). These characteristics were also found for B40 EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 (Tuinier, 1999). Xanthan is produced with a high efficiency, about 60 to 70% of the substrate is converted to polymer, and hence it is relatively cheap to produce this food thickener (Sutherland, 1998). The amount of EPS produced by *Lactococcus lactis* NIZO B40 in milk is low. We were able to increase the EPS production to 520 mg l^{-1} by optimising the culture conditions (Chapter 1). The highest efficiency for EPS production by strain NIZO B40 was under conditions of glucose limitation but under these conditions still only 2% of the substrate was converted to EPS (Chapter 3). For an industrial application of B40 EPS as a natural additive the efficiency of its production should be increased. Nevertheless, due to the fact that LAB are food grade organisms, the *in situ* production of polysaccharides remains also an interesting possibility and in that case low amounts of EPS could be sufficient to achieve desired properties. EPS-producing LAB are already successfully applied in the production of yoghurt in order to improve yoghurt stability and texture (Bouzar *et al.*, 1997; Hess *et al.*, 1997). The moisture and melting properties of low fat Mozzarella cheese could also be improved by the use of EPS-producing starter cultures (Perry *et al.*, 1997). The capsular polysaccharide of *Streptococcus thermophilus* MR-1C, which has a repeating unit

composed of galactose, rhamnose and fucose in a ratio of 5:2:1, was demonstrated to be responsible for this increased moisture retention (Low *et al.*, 1998). The increased water content of the low fat Mozzarella cheeses resulted not only in an improved quality of the cheeses but also provided an economic advantage. Using strain *Lactococcus lactis* NIZO B40 or other EPS-producing lactococci as starter cultures for the production of low fat (Gouda) cheese, it should be possible to increase moisture retention in the cheese. For this purpose however, it is a prerequisite that the EPS remains in the cheese matrix and is not removed with the whey in cheese manufacturing.

Although under acidifying conditions in milk, *Lactococcus lactis* NIZO B40 only produced a minor amount of EPS, this resulted in a significant increase in the viscosity of the fermented milk (Fig. 6.1). Milk supplemented with glucose and yeast extract which was fermented with the non-EPS-producing strain MG1614 was not viscous at all, whereas fermentation with the isogenic EPS-producing strain NZ4010 resulted in improved texture and increased viscosity (Fig. 6.1).

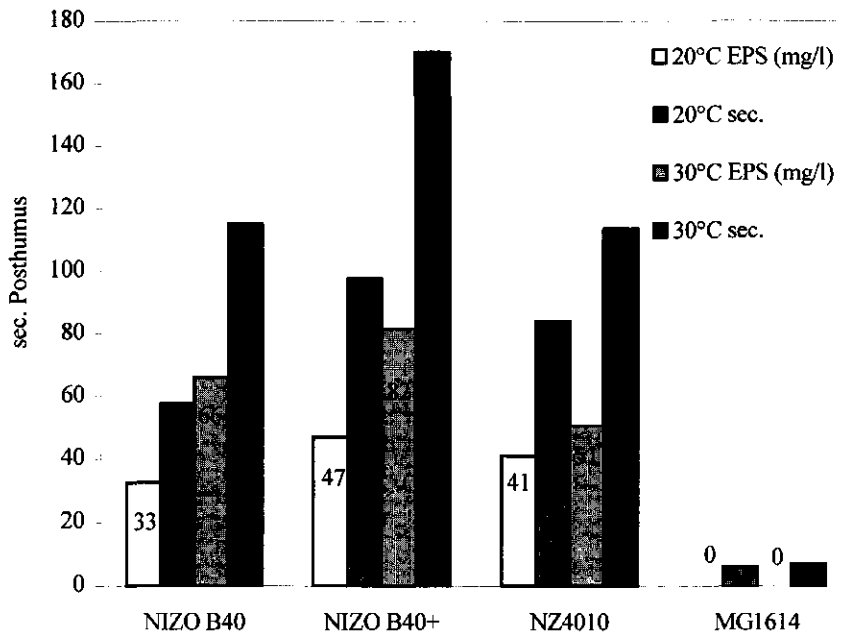

Figure 6.1

Viscosity and EPS concentration of milk fermented with *Lactococcus lactis* subp. *cremoris* at 20 and 30°C. Milk fermented with strain NZ4010, MG1614 and NIZO B40+ was supplemented with 2% glucose and 0.5% yeast extract. The viscosity was measured using a Posthumus funnel.

Biological activities

Furthermore, besides a positive influence on the rheological characteristics of (dairy) products, EPSs produced by LAB are claimed to have beneficial effects on the human health. These claims include cholesterol lowering capacity (Nakajima *et al.*, 1992), anti-tumour activity (Oda *et al.*, 1983; Kitazawa *et al.*, 1991) and immuno-potentiating effects (Nakajima *et al.*, 1995; Kitazawa *et al.*, 1996). The susceptibility of EPSs for digestion is an important feature in relation to their bioactive properties. Ruijssenaars *et al.* (2000) reported that EPS produced by *Lactococcus lactis* NIZO B40 is not susceptible to biological breakdown. In addition, we showed (Chapter 5) that EPS produced by *Lactococcus lactis* NIZO B40 resists digestion. The low amounts of EPS produced by *Lactococcus lactis* NIZO B40 in milk fermented with this organism might be enough for the exertion of any positive physiological effect. The EPS could for instance function as non-digestible food fibre. Based on structural similarities of the B40 EPS and the EPSs produced by the bacteria used in the studies of Kitazawa *et al.* (1996) and Nakajima *et al.* (1992) the B40 EPS could possibly also exert an immuno-potentiating or cholesterol lowering activity.

***Lactococcus lactis* as a cell factory for the production of EPS**

Immobilisation

We showed in Chapter 2 that EPS production and growth are not strictly coupled in *Lactococcus lactis* NIZO B40. This makes it possible to use the strain as an immobilised cell factory for the production of EPS while growth is prevented. A prerequisite for this system is that diffusion of EPS through the matrix is possible. Whey (permeate) could possibly be used as medium for this process. Whey contains enough sugar for EPS production but because strain NIZO B40 has limited proteolytic activity and a high demand of nutrients, it will probably not grow very well in whey. For subsurface isolates, attachment to solid surfaces resulted in enhanced exopolymer formation (Vandevivere and Kirchman, 1993). It is possible that immobilisation would also result in stimulation of the EPS production by *Lactococcus lactis*, but this has not been studied so far.

Reduction of lactate production

In fermentations with non-growing bacteria of *Lactococcus lactis*, EPS production was quite high during the first day but after that period the rate of EPS formation dropped quickly and EPS production and lactate formation stopped after several days although the sugar source was not exhausted. This could be caused by instability of the enzymes involved in EPS formation and/or general metabolism. These enzymes could not be synthesised during the experiments described in chapter 2 because chloramphenicol used to prevent growth of the bacteria stops protein synthesis. Apparently, some growth or protein synthesis is necessary to

maintain EPS synthesis for a longer period of time. It could be worthwhile to study if it is possible to use *Lactococcus lactis* as a cell factory for the production of EPS under these conditions by applying either a batch or a fill and draw type of fermentation. The fermentation fluid will be rich in lactate. Under several conditions we observed an uncoupling between growth and energy or lactic acid formation. The production of lactate could probably be reduced significantly without limitation of energy for EPS production. Reduction of the production of lactate will simplify the recovery of EPS from the fluid, not only because of a reduced lactate concentration but also because less titrant is used to control the pH and hence less salt needs to be removed. More important is that the reduction of the relative flux to lactate production could possibly result in an increase in the relative flux towards the direction of EPS biosynthesis. This could for example be achieved by using mutant strains with a reduced 6-phosphofructokinase or phosphoglucose isomerase activity.

Levels of sugar nucleotides

The EPS yield with fructose as the carbon source is strongly reduced compared with the yield on glucose (Chapter 2). In cultures grown on fructose the intracellular level of sugar nucleotides was much lower than the concentration of these EPS precursors with glucose as growth substrate. These reduced concentrations of sugar nucleotides, caused by a difference in the initial sugar metabolism, were shown to be responsible for the decreased EPS production and even for reduced growth on fructose (Chapter 4). Engineering the bacteria in such a way that the intracellular concentration of sugar nucleotides would increase, could possibly result in enhanced biosynthesis of EPS on other substrates as well. The natural concentrations of sugar nucleotides in the cells grown on glucose are quite high compared with growth on fructose. Overexpression of *epsD*, the gene involved in linking the first sugar of the repeating unit, resulted in a slight increase of the EPS production (van Kranenburg *et al.*, 1999). Nevertheless, an increase of the intracellular concentration of EPS-precursors by overexpression of the house-keeping enzymes involved in their production or repression of glycolytic enzymes (Fig. 6.2) could possibly result in a push into the direction of EPS production or even to a change of the sugar composition of the produced EPSs. This is presently under investigation (Boels *et al.*, 1999). Phosphoglucomutase (PGM) could be a key enzyme in the production of EPS as it links anabolism and catabolism (Fig. 6.2). In order to obtain information about which enzyme should be overexpressed or repressed to achieve higher intracellular concentrations of EPS-precursors or even higher EPS yields, modelling of the carbohydrate metabolism, including EPS production, might be useful. The modelling of the carbohydrate metabolism of *Lactococcus lactis* (Hoefnagel *et al.*, 2000) and studies on the regulation of the glycolytic flux in this organism are currently in progress (Andersen *et al.*, 2000; Koebmann *et al.*, 2000).

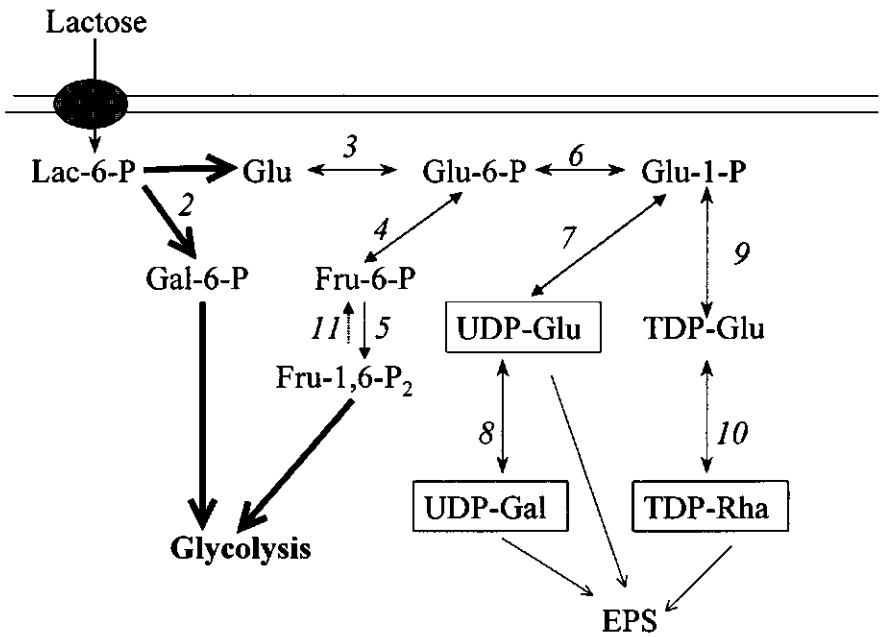

Figure 6.2

Simplified schematic representation of the metabolism of an EPS-producing *Lactococcus* cell grown on lactose. Lac: lactose, Glu: glucose, Fru: fructose, Gal: galactose, 1: lactose PEP-PTS, 2: phospho-β-galactosidase, 3: glucokinase, 4: phosphoglucose isomerase, 5: 6-phosphofructokinase, 6: α-phosphoglucomutase, 7: UDP-glucose pyrophosphorylase, 8: UDP-galactose-4-epimerase, 9: TDP-glucose pyrophosphorylase, 10: TDP-rhamnose biosynthetic enzyme system, 11: fructose-1,6-bisphosphatase.

Molecular mass and type of substrate limitation

In continuous cultures, the molecular mass of the EPS produced by *Lactococcus lactis* depends on the type of limitation. Under conditions of carbon limitation the molecular mass of EPS produced by strain NIZO B40 and NIZO B891 was strongly reduced compared to the EPS produced under conditions of nitrogen or phosphate limitation (Chapter 3). Imposing glucose limitation to continuous cultures of EPS-producing lactococci could possibly be used for the production of oligosaccharides. These oligosaccharides, which have only limited thickening properties, could for example be used as bodying agents with a high water binding capacity or as a non-digestible food fraction. In contrast to our expectations, the conversion of glucose to EPS was more efficient under conditions of glucose limitation than under nitrogen or phosphate limitation. Carbon-substrate limitation generally results in a high carbon conversion efficiency while the diversion of substrate carbon into extracellular products is

minimised in most organisms (Neijssel *et al.*, 1997). Under conditions of glucose limitation anabolism and catabolism are tightly balanced and for that reason it is very likely that the sugar source is more efficiently used for the formation of cell wall sugars. Probably, the biosynthesis of cell wall sugars and EPS are tightly coupled in growing bacteria explaining the higher efficiency of EPS production under glucose limitation.

Concluding remarks and suggestions for future research

Regulation of EPS production

EPSs produced by LAB influence the physical characteristics of (dairy) products and could even have beneficial effects on human health. The results described in this thesis are useful in view of optimisation of EPS production by *Lactococcus lactis*. The culture conditions and especially the sugar source had a major impact on the EPS yields. The reduced EPS production on fructose did not result from specific regulation but was caused by a difference in the initial sugar metabolism. In order to achieve a significant increase of the production of EPS, knowledge of the regulation mechanisms involved in EPS production could be helpful. Although we showed that EPS production can be uncoupled from growth (Chapter 2), the specific EPS production decreased strongly under conditions where growth was inhibited due to the presence of selective pressures in the environment such as increased oxygen tension, reduced water activity and the presence of copper ions (Chapters 2 and 5). The mechanisms involved in this negative influence on the EPS production could be the subject of further research. Furthermore, EPS productivity is normally highest at the mid-exponential growth phase and decreases thereafter. Some EPS is also produced during the stationary phase, but the rate of production is lower although at that moment the concentration of biomass is higher and there is no competition between growth and EPS production for sugar nucleotides and isoprenoid lipid carriers anymore. The high concentration of lactate in the medium might have a negative influence on the rate of EPS production. This could be a subject of future studies.

Physiological function of EPS

The physiological function of EPS produced by LAB is still not completely clear. We were able to find some protection of EPS against several anti-microbial factors but none of these factors could be used to increase the EPS production (Chapter 5). Plasmid pNZ4000 that encodes EPS production in *Lactococcus lactis* NZ4010 and NIZO B40 contains, besides the *eps* genes, nucleotide sequences that are homologous to genes involved in cobalt and magnesium transport. The combined action of these genes and the presence of EPS could possibly enhance the uptake of Co^{2+} and Mg^{2+} (van Kranenburg *et al.*, 2000). The possible role of EPS in sequestering these or other essential ions could be studied by comparing the minimal concentrations of the ions for growth of strain NZ4010, MG1614 and MG1614

harbouring plasmid pNZ4030. If EPS is involved in this transport, imposing Co^{2+} or Mg^{2+} limitation in continuous cultures might enhance EPS production.

Protection of the bacteria while passing through the gastro-intestinal tract, which is another possible function of EPS, was only tested *in vitro* where no difference in the survival between EPS-producing and non-producing bacteria was observed. Increase of the survival of bacteria during passage through the intestines due to the presence of EPS could be tested *in vivo* by feeding humans or animals milk fermented with either EPS-producing or non-producing strains.

Regulation of molecular mass

Under glucose limitation, EPS with a reduced molecular mass was produced (Chapter 3). We speculated that a reduced intracellular ATP concentration could be responsible for this phenomenon because one of the gene products involved in EPS production contains an ATP binding domain and is homologous to ExoP-like proteins, which are involved in chain-length determination (van Kranenburg *et al.*, 1997). The efficiency of EPS production was highest under glucose limitation. There could be a relationship between these two observations, a high conversion efficiency and a low molecular mass. Insight in the mechanism determining the actual size of the polymer might enable the production of EPSs with desired molecular masses.

REFERENCES

Andersen, H.W., Pedersen, M.B., Hammer, K. and Jensen, P.R. 2000. The importance of balanced expression of glycolytic genes in *Lactococcus lactis*. In: *Animating the cellular map*, Proceedings of the 9th International BioThermoKinetics Meeting, 3-8 April 2000, Stellenbosch, South-Africa.

Bouzar, F., Cerning, J. and Desmazeaud, M. 1997. Exopolysaccharide production and texture-promoting abilities of mixed-strain starter cultures in yoghurt production. J. Dairy Sci. **80:** 2310-2317.

Boels, I.C., Kleerebezem, M., Hugenholtz, J. and de Vos, W.M. 1999. Metabolic engineering of exopolysaccharide production in *Lactococcus lactis*. In: Book of abstracts, Sixth symposium on lactic acid bacteria, 19-23 September, Veldhoven, the Netherlands.

Hess, S.J., Roberts, R.F. and Ziegler, G.R. 1997. Rheological properties of nonfat yogurt stabilized using *Lactobacillus delbrueckii* ssp. *bulgaricus* producing exopolysaccharide or using commercial stabilizer systems. J. Dairy Sci. **80:** 252-263.

Hoefnagel, M.H.N., Hugenholtz, J., Westerhoff, H.V. and Snoep, J.L. 2000. Modelling pyruvate distribution in *Lactococcus lactis*: a kinetic model to support metabolic engineering strategies. In: *Animating the cellular map*, Proceedings of the 9th International BioThermoKinetics Meeting, 3-8 April 2000, Stellenbosch, South-Africa.

Kitazawa, H., Nomura, M., Itoh, T. and Yamaguchi, T. 1991. Functional alteration of macrophages by a slime-forming *Lactococcus lactis* ssp. *cremoris*. J. Dairy Sci. **74:** 2082-2088.

Kitazawa, H., Itoh, T., Tomioka, Y., Mizugaki, M. and Yamaguchi, T. 1996. Induction of IFN-γ and IL-1α production in macrophages stimulated with phosphopolysaccharide produced by *Lactococcus lactis* ssp. *cremoris*. Int. J. Food Microbiol. **31:** 99-106.

Koebmann, B.J., Pedersen, M.B., Nilsson, D. and Jensen, P.R. 2000. Is the glycolytic flux in *Lactococcus lactis* controlled by glycolysis itself? In: *Animating the cellular map,* Proceedings of the 9th International BioThermoKinetics Meeting, 3-8 April 2000, Stellenbosch, South-Africa.

Low, D., Ahlgren, J.A., Horne, D., McMahon, D.J., Oberg, C.J. and Broadbent, J.R. 1998. Role of *Streptococcus thermophilus* MR-1C capsular exopolysaccharide in cheese moisture retention. Appl. Environ. Microbiol. **64:** 2147-2151.

Morris, V.J. 1993. Fermentation-derived polysaccharides for use in foods. J. Chem. Technol. Biotechnol. **58:** 199-201.

Nakajima, H., Suzuki, Y., Kaizu, H. and Hirota, T. 1992. Cholesterol lowering activity of ropy fermented milk. J. Food Sci. **57:** 1327-1329.

Nakajima, H., Toba, T. and Toyoda, S. 1995. Enhancement of antigen-specific antibody production by extracellular slime products from slime-forming *Lactococcus lactis* subspecies *cremoris* SBT0495 in mice. Int. J. Food Microbiol. **25:** 153-158.

Neijssel, O.M., Snoep, J.L. and Teixeira de Mattos, M.J. 1997. Regulation of energy source metabolism in streptococci. J. Appl. Microbiol. **83:** 12S-19S.

Oda, M., Hasegawa, H., Komatsu, S. and Tsuchiya, F. 1983. Anti-tumor polysaccharide from *Lactobacillus* sp. Agric. Biol. Chem. **47:** 1623-1635.

Perry, D.B., McMahon, D.J. and Oberg, C.J. 1997. Effect of exopolysaccharide-producing cultures on moisture retention in low fat mozzarella cheese. J. Dairy Sci. **80:** 799-805.

Ruijssenaars, H., Stingele, F. and Hartmans, S. 2000. Biodegradability of food-associated extracellular polysaccharides. Curr. Microbiol. **40:** 194-199.

Sutherland, I.W. 1998. Novel and established applications of microbial polysaccharides. Tibtech **16:** 41-46.

Tuinier, R. 1999. An exocellular polysaccharide and its interactions with proteins. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Vandevivere, P. and Kirchman, D.L. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ. Microbiol. **59:** 3280-3286.

van Kranenburg, R., Vos, H.R., van Swam, I.I., Kleerebezem, M. and de Vos, W.M. 1999. Functional analysis of glycosyltransferase genes from *lactococcus lactis* and other Gram-positive cocci: Complementation, expression, and diversity. J. Bacteriol. **181:** 6347-6353.

Van Kranenburg, R., Kleerebezem, M. and de Vos, W.M. 2000. Nucleotide sequence analysis of the lactococcal EPS plasmid pNZ4000, Plasmid **43:** 130-136.

Summary

This thesis describes the results of a study of the physiology of exopolysaccharide (EPS) production by *Lactococcus lactis* subsp. *cremoris*. EPS produced by lactic acid bacteria are a potential source of natural additives for the use as thickening-, gelling- or stabilising agents in various food and non-food products. EPSs of lactic bacteria can also be produced *in situ* because lactic acid bacteria are food grade organisms. The model organism *L. lactis* subsp. *cremoris* NIZO B40 was used for most of the experiments. The genes necessary for EPS production by this strain are encoded on a 43-kb plasmid, pNZ4000. Whenever a good comparison between an EPS-producing and a non-producing strain was necessary, the EPS-producing strain NZ4010 and the non-EPS-producing plasmid-free strain MG1614 were used. Strain NZ4010 is a transconjugant of strain MG1614 carrying the *eps* plasmid pNZ4000. EPS produced by *L. lactis* NIZO B40 and NZ4010 is composed of glucose, galactose, rhamnose and phosphate.

The amount of EPS in milk fermented with strain NIZO B40 at 20°C is about 30 mg l^{-1}. We tried to increase this relatively low concentration by optimising the culture conditions and medium composition. A chemically defined medium was used for these experiments because it facilitates the study on the influence of individual components. The EPS yield of strain NIZO B40 greatly depended on the type of carbon source in the medium, glucose was the most efficient sugar source for EPS production. Cultivation under pH controlled conditions increased the amount of cell biomass and hence the concentration of EPS in the medium, but the amount of EPS produced per cell was also raised under these circumstances. Using such pH controlled conditions the influence of the culture pH and incubation temperature was investigated. The total EPS production was highest at pH 5.8 and 25°C. Under these conditions 520 mg of EPS per litre was produced. The EPS yield could not be improved any further by exposing the bacteria to stress conditions like a reduced water activity or an increased oxygen tension. The influence of the growth rate on EPS production was tested using chemostat cultures. Biosynthesis of structural cell polysaccharides and EPS both require energy, substrate, sugar nucleotides and isoprenoid lipid carriers. Due to a possible competition for the lipid carriers it is expected that under conditions of reduced growth the formation of EPS is increased due to an increased availability of lipid carriers. Within the range of 0.5 and 0.1 h^{-1}, reducing the growth rate resulted indeed in an increase of the specific EPS production but the polymer formation decreased again at a growth rate of 0.05 h^{-1}. Energy supply could have been the limiting factor for EPS production at this growth rate.

Most culture conditions influence growth as well as EPS formation and EPS synthesis itself was also shown to be influenced by the growth rate. For that reason we studied the linking between growth and EPS synthesis. EPS production by NIZO B40 starts at the exponential growth phase but continues during the stationary phase, which indicated that EPS

biosynthesis and growth are not strictly coupled in this organism. Indeed we found that non-growing cultures were still able to produce EPS. This finding made it possible to study the influence of different culture conditions on EPS biosynthesis independent of growth.

The optimal temperature for EPS production by non-growing cultures of NIZO B40 was 25°C. Reduction of the water activity resulted in a decrease of the EPS production by the non-growing cultures as was also found for growing cultures. The sugar composition of EPS was unaffected by the sugar source, growth rate or culture conditions (Chapter 2).

Growth and EPS production both require a substrate for the supply of sufficient energy and for the biosynthesis of biomass and EPS precursors. Amino acids are necessary for growth but are not directly involved in EPS production. For that reason it is generally assumed that EPS production will increase under conditions such as amino acid limitation in the presence of excess carbohydrates. Nevertheless, the amounts of EPS produced by *L. lactis* NIZO B40 and NIZO B891 were comparable under glucose and leucine limitation. The efficiency of EPS production, which is defined as the amount of EPS produced per amount of glucose that was consumed, was however much higher under conditions of glucose limitation. The production of phosphorylated EPS by strain NIZO B40 as well as the production of unphosphorylated EPS by strain NIZO B891 were strongly reduced under conditions of phosphate limitation compared with glucose and leucine limitation. The sugar composition of both B40 and B891 EPS and the phosphate content of B40 EPS were unaffected by the type of limitation but surprisingly, the type of limitation had a remarkable influence on the molecular mass of the EPSs. Glucose limitation resulted in the production of EPSs with a strongly reduced molecular mass compared to EPSs produced under leucine and phosphate limitation. This resulted in a marked decrease of the intrinsic viscosity of EPSs produced under conditions of glucose limitation (Chapter 3).

Lactococcus lactis subsp. *cremoris* NIZO B40 produces about 9 times more EPS with glucose than with fructose as a sugar source under acidifying conditions. Regulation of EPS production by the sugar source could be possible at all the different steps involved in its biosynthesis: substrate uptake, formation of repeating units, export of the repeating units and polymerisation of the repeating units. Glycosyltransferases, necessary for the formation of the repeating units and the enzymes involved in export and polymerisation of the repeating units are plasmid encoded. The *eps* gene cluster of these enzymes is transcribed from a single promoter. The activity of this promoter was shown to be independent of the type of sugar in the medium. A major difference between glucose and fructose grown bacteria was the intracellular concentration of UDP-glucose and UDP-galactose, which was much lower in the fructose grown bacteria. These two sugar nucleotides and TDP-rhamnose are EPS-building blocks as well as precursors for the synthesis of cell wall polysaccharides. The activity of the enzymes involved in the biosynthesis of the sugar nucleotides were unaffected by the source of sugar but the activity of fructose-1,6-bisphosphatase (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. FBPase catalyses the conversion of fructose-1,6-diphosphate into fructose-6-phosphate which is an essential step for the biosynthesis of sugar nucleotides from fructose but not from glucose. Overexpression of the *fbp* gene resulted in increased EPS synthesis on fructose. Apparently, the activity of this enzyme limits the amount of EPS produced by wild type *L. lactis* subsp. *cremoris* on fructose and hence the initial sugar metabolism was responsible for the difference in EPS yield on fructose and glucose (Chapter 4).

Finally, the physiological function of EPS produced by lactic acid bacteria was studied in order to find tools for improvement of the relatively low EPS production yields or to obtain insight into the regulation mechanisms that are involved in EPS biosynthesis by *L. lactis*. In order to find the physiological function of EPS produced by *L. lactis* subsp. *cremoris*, the sensitivity of an EPS-producing strain and its non-producing isogenic parent strain to various anti-microbial factors was compared. EPS produced by the ropy strain, could not be used as an energy source but it protected the bacteria against several anti-microbial factors. Cell–associated EPS resulted in a decrease of the sensitivity of the bacteria to bacteriophages and lysozyme, probably by masking the targets for the phages and the enzyme. Cell-associated EPS as well as EPS in suspension protected the bacteria against toxic copper ions and the lantibiotic nisin. The protective mechanism in these cases is most likely based on charge interactions between the anionic EPS and the positively charged nisin and copper ions. EPS had no influence on the survival of bacteria when exposed to increased temperatures, freezing, freeze-drying, penicillin, vancomycin and digestion. The EPS itself was not degraded during passage through the gastro-intestinal tract of rats. The protection of *L. lactis* NZ4010 EPS against nisin and bacteriophages could be a competitive advantage in mixed strain dairy starter cultures. Unfortunately, the EPS yields were not increased in the presence of copper, bacteriophages, nisin or lysozyme (Chapter 5).

The work described in this thesis is discussed and suggestions for future research are given (chapter 6).

Samenvatting

Dit proefschrift beschrijft de resultaten van onderzoek naar de vorming van exopolysachariden (EPS) door de melkzuurbacterie *Lactococcus lactis* subsp. *cremoris* NIZO B40. De term exopolysachariden bestaat uit 3 delen: sachariden (=suikers), poly (=veel) en exo (=buiten). Exopolysachariden zijn dus moleculen bestaande uit honderden of duizenden aan elkaar gekoppelde suikers, die door bacterie in hun leefmilieu worden uitgescheiden. *Lactococcus lactis* is een melkzuurbacterie die door de zuivelindustrie veelvuldig wordt gebruikt voor de gewenste omzetting van melksuiker in melkzuur in producten als kaas, kwark, zure room en karnemelk. Deze omzetting zorgt er voor dat de producten zuur worden en de houdbaarheid wordt vergroot. Daarnaast zijn de melkzuurbacteriën door omzetting van eiwit uit de melk in aromacomponenten ook deels verantwoordelijk voor de smaak van zuivelproducten. Als bijproducten bij de omzetting van suikers, worden door een aantal melkzuurbacteriën EPSen gemaakt, eiwitten die de groei van bacteriën remmen, de zgn. bacteriocines, gevormd of worden specifieke aromacomponenten geproduceerd. EPSen hebben over het algemeen een sterke invloed op de rheologische eigenschappen van producten en kunnen daarom gebruikt worden als verdikkings- of geleermiddel in allerlei (voedings)producten. Omdat het veilig is om melkzuurbacteriën te consumeren is het mogelijk de verdikkende stoffen, de EPSen, door de bacteriën in de producten zelf te laten vormen. Het is echter ook mogelijk om de EPSen als natuurlijk additief aan producten toe te voegen. Dit laatste wordt al veelvuldig gedaan met EPSen zoals xanthaan en gelaan, die gemaakt worden door bacteriën die zelf niet in voedingsmiddelen mogen voorkomen (Hoofdstuk 1).

EPS geproduceerd door stam NIZO B40 is opgebouwd uit de bouwstenen glucose, galactose, rhamnose en fosfaat. In melk wordt door deze bacterie bij 20°C slechts 30 mg EPS per liter geproduceerd. Wij hebben geprobeerd deze relatief lage hoeveelheid te verhogen door te onderzoeken welke kweekcondities en welke samenstelling van het groeimedium optimaal zijn voor de vorming van EPS. Hiervoor hebben we gebruik gemaakt van een chemisch gedefinieerd medium waardoor het makkelijker is de invloed van specifieke mediumcomponenten te onderzoeken. De suikerbron in het medium had een grote invloed op de hoeveelheid EPS die werd geproduceerd, glucose was de meest efficiënte suikerbron voor EPS-productie. Door de pH van het medium tijdens de kweekproeven constant te houden kon een hogere bacterieconcentratie gerealiseerd worden en namen zowel de totale EPS-productie als de hoeveelheid EPS geproduceerd per bacterie sterk toe. De hoeveelheid EPS kon verhoogd worden tot 520 mg per liter door de bacteriën te kweken bij een constante pH van 5.8 en een temperatuur van 25 °C in chemisch gedefinieerd medium met 60 gram glucose per liter. Blootstelling van de bacteriën aan stresscondities resulteerde niet in een verdere verhoging van de EPS-productie. De invloed van de groeisnelheid op de EPS-productie werd

onderzocht door gebruik te maken van continu cultures waarbij een relatief lage concentratie van een specifiek component in het medium, dat constant aan het kweekvat wordt toegevoerd, bepaalt hoe snel de bacteriën kunnen groeien. De vorming van polysachariden die voorkomen in de celwanden van de bacteriën en de vorming van EPS zijn allebei afhankelijk van de aanvoer van energie, de vorming van bouwstenen en de aanwezigheid van hulpmiddelen zoals de zgn. lipide carriers. Door een mogelijke competitie voor deze intermediairen wordt verwacht dat onder kweekcondities waarbij de bacteriën minder snel groeien en dus minder celwand polysachariden gemaakt hoeven te worden, meer van deze carriers beschikbaar zijn voor EPS-vorming met als gevolg een verhoogde productie van EPS. Tot op zekere hoogte resulteerde verlaging van de groeisnelheid inderdaad in een verhoogde EPS-productie. Wanneer de groeisnelheid echter nog verder werd verlaagd dan nam de hoeveelheid EPS die per bacterie werd gevormd weer af, mogelijk door gebrek aan energie voor de productie van EPS.

De meeste kweekcondities beïnvloeden zowel de groei als de EPS-vorming en ook de EPS-productie zelf bleek afhankelijk te zijn van de groeisnelheid. We hebben aangetoond dat niet-groeiende bacteriën in staat waren om EPS te vormen. Deze waarneming maakt het mogelijk om de invloed van kweekcondities e.d. op de EPS-productie, onafhankelijk van groei, te onderzoeken. De chemische samenstelling van het EPS gevormd door stam NIZO B40 werd niet beïnvloed door de kweekcondities, de mediumsamenstelling of door het gebruik van niet-groeiende bacteriën (Hoofdstuk 2).

Voor zowel groei als EPS-vorming hebben de bacteriën een suikerbron nodig om de nodige energie en bouwstenen aan te leveren. Andere componenten uit het medium zoals aminozuren en fosfaat zijn wel nodig voor de groei maar zijn niet direct betrokken bij EPS-productie. Hierdoor werd er verwacht dat onder condities waar de groei beperkt wordt door een limiterende concentratie aminozuren of fosfaat in de aanwezigheid van een overmaat suikerbron, meer EPS gevormd zou worden. Dit was echter niet het geval voor de stammen *L. lactis* NIZO B40 en NIZO B891. De efficiëntie van de EPS-productie, d.w.z de hoeveelheid EPS geproduceerd per hoeveelheid glucose die werd geconsumeerd, was juist veel hoger onder condities waar de groei beperkt werd door een lage concentratie glucose in het medium. Opvallend was dat het molecuulgewicht, ofwel het aantal aan elkaar gekoppelde suikers, sterk afnam onder deze condities waardoor de verdikkende werking van het EPS sterk afnam (Hoofdstuk 3). .

Lactococcus lactis NIZO B40 produceert ongeveer 9 keer zoveel EPS wanneer glucose als suikerbron in het medium aanwezig is dan wanneer fructose als suikerbron wordt gebruikt. We hebben onderzocht waardoor dit veroorzaakt wordt. De concentratie in de cel van geactiveerde suikers, de bouwstenen voor zowel EPS als celwand polysachariden, bleek veel lager te zijn bij groei in medium met fructose dan in medium met glucose. Dit werd veroorzaakt door een erg lage activiteit van het enzym fructosebisfosfatase. Dit enzym is wel

nodig voor de vorming van de EPS-bouwstenen wanneer fructose als suikerbron wordt gebruikt maar niet wanneer glucose als suikerbron in het medium voorkomt (Hoofdstuk 4).

Tenslotte werd de functie van EPS voor de melkzuurbacteriën die het EPS produceren onderzocht met als achterliggende gedachte het vinden van mogelijkheden om de productie te verhogen en om inzicht te krijgen in de regulatie van de EPS-vorming. Om de functie van EPS te vinden werd de gevoeligheid van gelijksoortige bacteriën die wel en die niet in staat waren om EPS te produceren voor allerlei bacteriegroei remmende factoren met elkaar vergeleken. Een laag EPS rondom de bacteriecellen zorgde voor bescherming van de bacteriën tegen de aanval van bacteriofagen en tegen lysozym, een enzym dat in staat is om de celwanden van bacteriën af te breken. De aanwezigheid van EPS rondom de cellen alsmede de aanwezigheid van EPS in het medium beschermden de bacteriën tegen giftige koper ionen en het bacteriocine nisine. De beschermende werking van EPS is in dit geval waarschijnlijk gebaseerd op ladingsinteracties. Het negatief geladen EPS vangt vermoedelijk de positief geladen koper en nisine moleculen weg. EPS had geen invloed op de overleving van de bacteriën wanneer deze werden blootgesteld aan verhoogde en verlaagde temperaturen, aan drogen of verschillende antibiotica. EPS zelf werd niet afgebroken tijdens de passage door het maagdarm kanaal van ratten maar de aanwezigheid van EPS beschermde de bacteriën niet tegen vertering. Helaas kon de EPS-productie niet verhoogd worden door blootstelling van de producerende organismen aan koper, nisine, bacteriofagen of lysozym (Hoofdstuk 5).

Het werk dat is beschreven in dit proefschrift is bediscussieerd en er zijn suggesties gedaan voor toekomstig onderzoek in hoofdstuk 6.

Nawoord

Het schrijven van deze laatste pagina's heb ik zolang mogelijk uitgesteld, maar nu op deze regenachtige vrije dag moet het er toch maar eens van komen. Er wordt meestal verondersteld dat je hier terugblikt op een geweldige periode en iedereen bedankt, die ook maar enigszins behulpzaam is geweest bij de succesvolle afronding van het promotie-onderzoek. Natuurlijk wil ook ik zeker aan dit laatste punt niet voorbijgaan. Maar laat ik eerst eens vertellen hoe het allemaal is begonnen.

Voordat ik aan een stage bij NIZO begon, die begeleid werd door de AIO Wilco Meijer, was het nog geen moment in me opgekomen om zelf ook ooit AIO te worden. Ik vond ook absoluut niet dat ik daar geschikt voor was. Vooral het geven van presentaties, de begeleiding van stagiaires en het schrijven van publicaties leken me onmogelijke taken. Wilco wist de meeste van mijn argumenten om vooral geen AIO te worden wat af te zwakken en toen vlak na mijn afstuderen het aanbod kwam om AIO te worden bij NIZO, heb ik dan ook ja gezegd. Ik zag er toch ook wel de voordelen van in. Een stage of afstudeervak duurde altijd te kort. Op het moment dat het juist allemaal interessant begon te worden, zat de tijd erop. Vier jaar lang aan één onderwerp werken leek me daarom wel wat. En eerlijk is eerlijk, de arbeidsmarkt was op dat moment ook niet zo geweldig.

Ik ben lang niet altijd blij geweest met deze beslissing. Heel lang heb ik ook gedacht dat dit boekje er nooit zou komen. Vooral de experimenten heb ik echter met veel plezier uitgevoerd en ik had deze periode eigenlijk ook zeker niet willen missen. En daarmee kom ik dan bij de mensen die hierbij een belangrijke rol hebben gespeeld.

In de eerste plaats mijn begeleiders. In het begin was dit vooral Mark Smith, die er voor gezorgd heeft dat mijn onderzoek een beetje op gang kwam. Na het vertrek van Mark naar zijn geboorteland, werd de begeleiding overgenomen door Jeroen, die ik vooral wil bedanken voor de tips bij het schrijven van de laatste stukken tekst en voor het feit dat ik mijn onderzoek redelijk zelfstandig kon uitvoeren. Ook mijn promotor Jan heeft eigenlijk pas in een later stadium een rol gespeeld. Jan, bedankt voor je inzet en vooral ook bedankt dat je toch mijn promotor wilde zijn.

De begeleiding van stagiaires vond ik uiteindelijk toch één van de leukere taken van een AIO. Een deel van het werk, dat in dit proefschrift is beschreven, is dan ook uitgevoerd door stagiaires. Hester, Manuela, en Eric, hartelijk dank voor alle experimenten die jullie hebben gedaan. Chantal, ik ben blij met al het werk dat je hebt uitgevoerd maar vooral voor het feit dat onze vriendschap niet onder je stage heeft geleden. Lionel, it was not always easy to work with such a stubborn, chauvinistic Frenchman, especially not when France won the World Championship, but in the end all I can say is: "en un mot: merci".

Collega's zijn erg belangrijk voor me geweest. Eerst waren er de mensen van de afdeling microbiologie waarbij vooral Fedde en Marjo een speciaal woord van dank verdienen. Fedde

heeft me, door al zijn werk aan EPS geproduceerd door yoghurt-bacteriën, behoorlijk op weg geholpen bij het gebruik van allerlei methoden. Marjo was een altijd geduldige vraagbaak bij het uitvoeren van mijn vele fermentaties. Ook alle andere personen uit mijn directe werkomgeving, Annemarie, Esther, Bert, Renate, Hans, Arjen, Marja, Leo, Ad en Zeger, en overige microbiologen wil ik bedanken voor de prettige werksfeer. Ik heb genoten van alle badminton avondjes.

Na microbiologie kwam MI. Eerst had ik helemaal geen zin om naar de kelder te verhuizen, maar al gauw bleek dat het daar minstens zo gezellig was als boven, waarvoor ik alle MItjes dan ook van harte wil bedanken. Van de NIZO EPS-groep wil ik vooral Richard, Ingeborg en Remco bedanken omdat ze steeds weer bereid waren om stukken tekst door te lezen. Roellie, Jan en Cees, van analytische chemie, ook jullie bedankt voor alle hulp. De andere AIO's en hun begeleiders van het ABON project waartoe ook mijn onderzoek behoorde bedank ik hierbij voor de samenwerking en de vele nuttige suggesties.

Tijdens mijn studie en tijdens de promotie-tijd heb ik steeds veel steun gehad van mijn ouders. Evenals andere familieleden en vrienden zijn dat dan ook de belangrijkste personen om hier te bedanken. Het was lang niet altijd gemakkelijk om iedereen uit te leggen waar ik nou eigenlijk mee bezig was. De keren dat ik vragen kreeg als 'wanneer studeer je af', 'is je verslag al af' en 'ben je nu nog niet klaar met school' waren ontelbaar maar steeds was er een ruime belangstelling voor wat ik deed.

Arjan ook jij enorm bedankt. Niet alleen voor de ontelbare keren dat je 's avonds of in het weekend toch maar weer even met me naar NIZO reed, omdat ik nog een monstertje wilde nemen of twijfelde of de loog niet op zou raken etc., maar vooral ook voor je mentale steun. Als ik het even niet zag zitten wist jij me wel af te leiden of weer te motiveren, juist door te zeggen: "als je er geen zin meer in hebt dan stop je er toch gewoon mee, we redden het evengoed wel".

Curriculum vitae

Petronella Johanna (Ellen) Looijesteijn werd op 25 februari 1969 geboren in de gemeente Anna Paulowna. In 1987 behaalde zij het VWO diploma aan het Johannes College te Den Helder en aansluitend begon zij met de opleiding Levensmiddelentechnologie aan de Hogere Agrarische School in Bolsward, met als afstudeerrichting Voeding en Kwaliteit. Tijdens deze studie heeft zij stages uitgevoerd bij de Melkunie in Lutjewinkel (algemene laboratorium werkzaamheden), Eyssen smeer- en smeltkaasfabriek in Oosthuizen (schrijven van mannuals), de AHoF in Bolsward (optimalisatie reiniging en desinfectie) en bij Waterschap Regge en Dinkel in Oldenzaal (onderzoek i.v.m lichtslibbestrijding). De studie werd afgerond met een afstudeeropdracht gericht op de ontwikkeling van een alcoholische weidrank voor een biologisch dynamisch bedrijf. Na het behalen van haar diploma stroomde ze door naar de Landbouwuniversiteit te Wageningen, studie Levensmiddelentechnologie. In het kader van deze studie deed zij een afstudeervak bij de sectie levensmiddelenmicrobiologie (fysiologie van *Listeria monocy*togenes) en een vrijwillige onderzoeksstage bij NIZO in Ede (lysis van *Lactococcus lactis*). De studie werd *cum laude* afgesloten in augustus 1994. Kort hierna is zij gestart met haar promotie-onderzoek, dat werd uitgevoerd bij NIZO food research in Ede onder begeleiding van dr. J. Hugenholtz. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Momenteel heeft zij een tijdelijke betrekking bij Borculo Domo Ingredients in Zwolle en is daar werkzaam binnen de afdeling Quality Assurance.

List of publications

Looijesteijn, P.J. and Hugenholtz, J. 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40. J. Biosci. Bioeng. **88:** 178-182.

Looijesteijn, P.J., Boels, I.C., Kleerebezem, M. and Hugenholtz, J. 1999. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. **65:** 5003-5008.

Kleerebezem, M., van Kranenburg, R., Tuinier, R., Boels, I.C., Zoon, P., Looijesteijn, E., Hugenholtz, J. and de Vos, W.M. 1999. Exopolysaccharides produced by *Lactococcus lactis*: from genetic engineering to improved rheological properties? Antonie van Leeuwenhoek **76:** 357-365.

Looijesteijn, P.J., van Casteren, W.H.M., Tuinier, R., Doeswijk-Voragen, C.H.L. and Hugenholtz, J. 2000. Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures. J. Appl. Microbiol. **89:** 116-122.

Hugenholtz, J., Looijesteijn, E., Starrenburg, M. and Dijkema, C. 2000. Analysis of sugar metabolism in an EPS-producing *Lactococcus lactis* by ^{31}P NMR. J. Biotechnol. **77:** 17-23.

Looijesteijn, P.J., Trapet, L., de Vries, E., Abee, T. and Hugenholtz, J. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int. J. Food Microbiol., in press.

Tuinier, R., van Casteren, W.H.M., Looijesteijn, P.J., Schols, H.A., Voragen, A.G.J. and Zoon, P. Structure-function relationship of (modified) EPSs from *Lactococcus lactis*. Submitted.

Physiology of exopolysaccharide biosynthesis by *Lactococcus lactis*

Ellen Looijesteijn

Promotor: Dr. Ir. J.A.M. de Bont
Hoogleraar in de industriële microbiologie

Co-promotor: Dr. J. Hugenholtz
Projectleider afdeling "Flavour & Natural Ingredients"
NIZO food research, Ede

Stellingen

1. Het gebruik van viscositeitsmetingen om exopolysacchariden te kwantificeren (Gassem *et al.*, 1997), is riskant omdat kweekcondities een invloed kunnen hebben op het molecuulgewicht van de gevormde exopolysacchariden (Degeest and de Vuyst, 1999; dit proefschrift).
   Gassem, M.A., Schmidt, K.A. and Frank, J.F. 1997. J. Food Sci. **62:** 171-173.
   Degeest, B. and de Vuyst, L. 1999. Appl. Environ. Microbiol. **65:** 2863-2870.

2. De slechte afbreekbaarheid van B40 EPS (Ruijssenaars *et al.*, 2000) doet vermoeden dat dit polymeer bij consumptie dezelfde gezondheidseffecten heeft als andere voedingsvezels, die niet afbreekbaar zijn.
   Ruijssenaars, H.J., Stingele, F. and Hartmans, S. 2000. Curr. Microbiol. **40:** 194-199.

3. Het vervangen van ammoniumcitraat door sodiumcitraat bij het kweken van *Lactococcus lactis* subsp. *cremoris* LC330 onder stikstoflimitatie door Marshall *et al.* (1995) is een overbodige variatie omdat dit organisme niet in staat is anorganisch stikstof als stikstofbron te gebruiken (Cocaign-Bousquet *et al.*, 1995).
   Marshall, V.M., Cowie, E.N. and Moreton, R.S. 1995. J. Dairy Res. **62:** 621-628.
   Cocaign-Bousquet, M., Garrigues, C., Novak, L., Lindley, N.D. and Loubiere, P. 1995. J. Appl. Bacteriol. **79:** 108-116.

4. Het doorstralen van voedsel is een veilige methode om pathogenen te doden, maar mag geen alternatief zijn voor een hygiënische voedselproductie.
   Lutter, R. 1999. Science **286:** 2275-2276.
   Wood, O.B. and Bruhn, C.M. 2000. J. Am. Diet. Assoc. **100:** 246-253.

5. Veel personen denken ten onrechte dat ze lactose-intolerant zijn, maar ook indien de diagnose daadwerkelijk lactose-intolerantie is, behoeven zuivelproducten niet uit het menu geschrapt te worden.
   L.D. McBean, G.D. Miller (1998) J. Am. Diet. Assoc. **98:** 671-676.

6. HACCP-studies leiden niet tot het uitbannen van exotoxinen en virussen in voedsel.
   Centraal college van deskundigen-HACCP. 1998.
   Fleet, G.H., Heiskanen, P., Reid, I. and Buckle, K.A. 2000. Int. J. Food Microbiol **59:** 127-136.

7. Bij Heinz steken ze de draak met de wet van behoud van massa.
   Etiket Sandwich Spread tomaat lente-ui: 101g tomaten per 100g product.

8. Werken in de horeca is gezonder dan werken in de gezondheidszorg.
   CBS. 2000. Persbericht 00-099.

9. Het veroorzaakt regelmatig verwarring wanneer de eerste letter van je doopnaam niet overeenkomt met die van je roepnaam. Toekomstige ouders zouden hier rekening mee 'moeten' houden.

Stellingen behorende bij het proefschrift
"Physiology of exopolysaccharide biosynthesis by *Lactococcus lactis*"
Ellen Looijesteijn
Wageningen, 22 november 2000