Exopolysaccharides produced by Lactococcus lactis : from genetic engineering to improved rheological properties?

Antonie van Leeuwenhoek **76:** 357–365, 1999.

Exopolysaccharides produced by *Lactococcus lactis*: from genetic engineering to improved rheological properties?

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Abstract

Over the last years, important advances have been made in the study of the production of exopolysaccharides (EPS) by several lactic acid bacteria, including *Lactococcus lactis*. From different EPS-producing lactococcal strains the specific *eps* gene clusters have been characterised. They contain *eps* genes, which are involved in EPS repeating unit synthesis, export, polymerisation, and chain length determination. The function of the glycosyltransferase genes has been established and the availability of these genes opened the way to EPS engineering. In addition to the *eps* genes, biosynthesis of EPS requires a number of housekeeping genes that are involved in the metabolic pathways leading to the EPS-building blocks, the nucleotide sugars. The identification and characterisation of several of these housekeeping genes (*galE, galU, rfbABCD*) allows the design of metabolic engineering strategies that should lead to increased EPS production levels by *L. lactis*. Finally, model development has been initiated in order to predict the physicochemical consequences of the addition of a EPS to a product.

Microbial exopolysaccharides

Polysaccharides are a highly diverse group of polymers, of which the functional features are determined by their structural characteristics that may differ in molecular weight, saccharide-linkage type, degree of branching and chemical composition. This diversity has led to very broad application possibilities in industry, ranging from paper manufactury to oil recovery and food production. High molecular weight polysaccharides are indispensable ingredients in a large variety of food products, where they are used as thickeners, stabilisers, emulsifiers, fat-replacers or gelling agents. Currently, most of the polysaccharides used in food-industry are derived from plants (e.g. starch, pectin, cellulose) and seaweeds (e.g. alginate, carrageenan). Alternative sources of biothickeners are found among the microbial extracellular polysaccharides (Sutherland 1998), which can either be present as capsular polysaccharide (CPS) associated with the cell surface or secreted as extracellular polysaccharide (EPS) in the environment of the cell. Four classes of microbial polysaccharides can be distinguished on the basis of their composition and mechanism of biosynthesis (Sutherland 1993). In this review we will only discuss the heteropolysaccharides that consist of repetitive, regular repeating units. In terms of application in food products, one of the most important examples of this class of bacterial polysaccharides is xanthan, which is produced by the phytopathogenic bacterium *Xanthomonas campestris*. Despite the fact that *X. campestris* is not an acceptable bacterium in food products (food-grade), the rheological properties of xanthan have led to its wide application as food-additive. Preferentially, bacterial polysaccharides to be used as additives in food products should be produced by non pathogenic, 'safe' bacteria. Lactic acid bacteria (LAB) in general have a 'food-grade' status and EPSs produced by these bacteria can be considered as 'food-grade' additives. Therefore, EPS production by LAB has received considerable attention since they appear to be relevant for dairy-product properties like texture and mouthfeel. Moreover, it has been suggested that they are active as prebiotic (Gibson & Robertfroid 1995), cholesterol lowering neutraceutical (Nakajima et al. 1992a) or immun-

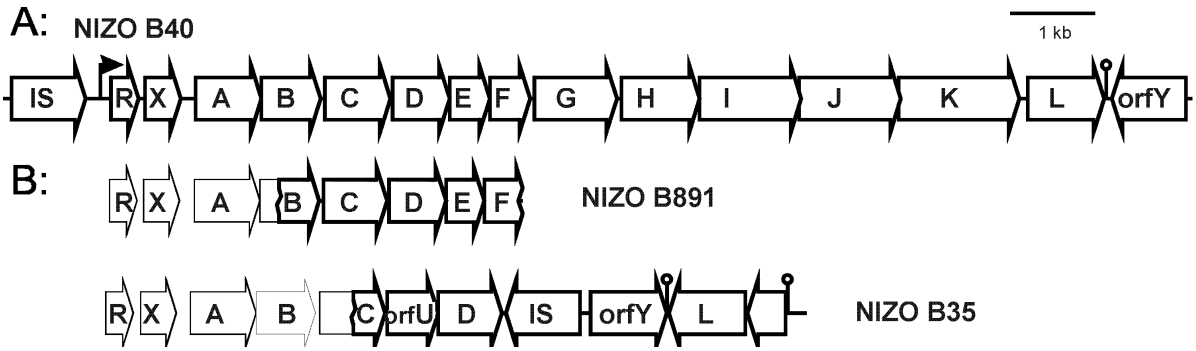

Figure 1. Genetic organisation of the *L. lactis* NIZO B40 (A), B891 (B) and B35 (B) *eps* gene cluster. In Figure 2B, PCR-product based gene annotations are indicated as thin arrows while sequences and annotations derived from cloned regions are indicated as bold arrows (van Kranenburg et al. 1999b).

omodulant (Kitazawa et al. 1993; Hosono et al. 1997). Over the last years, the chemical structure, and the molecular biology and genetics of the biosynthesis, of various repetitive heteropolysaccharide EPSs produced by LAB have been studied (Stingele et al. 1996; Stingele 1998; van Kranenburg et al. 1997, 1999a,b; Lamothe et al. 1998). Here, we review research on EPS production by the mesophilic LAB *Lactococcus lactis*. Both EPS-specific genetics and the general metabolism that is required for EPS precursor biosynthesis will be discussed. Finally, we will give an impression of recent advances of model-forming research considering the physicochemical properties of EPS in watery solution and its physical interactions with milk components.

EPS genetics in Lactococcus lactis

The biosynthesis of EPSs that consist of repetitive, regular repeating units appears to follow a common mechanism. The repeating units are assembled at the membrane by the sequential addition of sugars to the growing repeating unit that is anchored on the lipid carrier. After completion of an EPS repeating unit, it is supposed to be exported and polymerised to form the cell-surface polysaccharides (Sutherland 1993). The production of EPS by *L. lactis* is associated with strains that are isolated from highly viscous Scandinavian fermented milk products. The EPS specific *eps* genes are encoded on large plasmids (>20 kb) that can be transferred from one lactococcal strain to the next by conjugation, which concomitantly results in transfer of EPS production (Vedamuthu and Neville 1986; von Wright and Tynkkynen 1987; van Kranenburg et al. 1997, 1998, 1999a,b). The best characterised EPS producing *L. lactis* strain is NIZO B40 that produces a phosphopolysaccharide that is structurally identical to that produced by *L. lactis* strain SBT 0495, with a repeating unit consisting of $\rightarrow$4)[α-L-Rha*p*-(1$\rightarrow$2)][α-D-Gal*p*-1-PO_4-3]-β-D-Gal*p*-(1$\rightarrow$ 4)-β-D-Glc*p*-(1$\rightarrow$4)-β-D-Glc*p*-(1$\rightarrow$ (Figure 2) (Nakajima et al. 1990, 1992b; van Kranenburg et al. 1997, 1999a). A 12 kb gene cluster that is specific for NIZO B40 EPS production contains 14 coordinately expressed genes, *epsRXABCDEFGHIJKL* (Figure 1A), and is localised on a 40 kb plasmid designated pNZ4000 (van Kranenburg et al. 1997). Based on homology to gene products encoded by other polysaccharide biosynthetic gene clusters, putative functions in polysaccharide export, polymerisation, chain length determination and subunit biosynthesis (glycosyltransferase; GTF) could be assigned to several gene products encoded within the B40 gene cluster (Figure 1A; van Kranenburg et al. 1997). An interesting (see also below) feature like chain length determination putatively depends on the activity of EpsA and EpsB, which share overall homology and specific consensus motifs with the family of ExoP-like proteins. ExoP is supposed to be involved in chain length determination of succinoglycan produced by *R. meliloti* (Becker et al. 1995). The homologous counterparts of the EpsK and EpsI play a role in *Salmonella* or *Shigella* O-antigen production as a lipid-linked repeating unit flippase-exporter and polymerase, respectively (Morona et al. 1994; MacPherson et al. 1995; Liu et al. 1996). The function of the B40-GTF encoding genes was determined *in vitro* using heterologous (*E. coli*) and homologous (non-EPS producing *L. lactis*) inducible expression of these genes. Induced cells were permeabilized and incubated with UDP-[^{14}C]-glucose or UDP-[^{14}C]-galactose, followed by

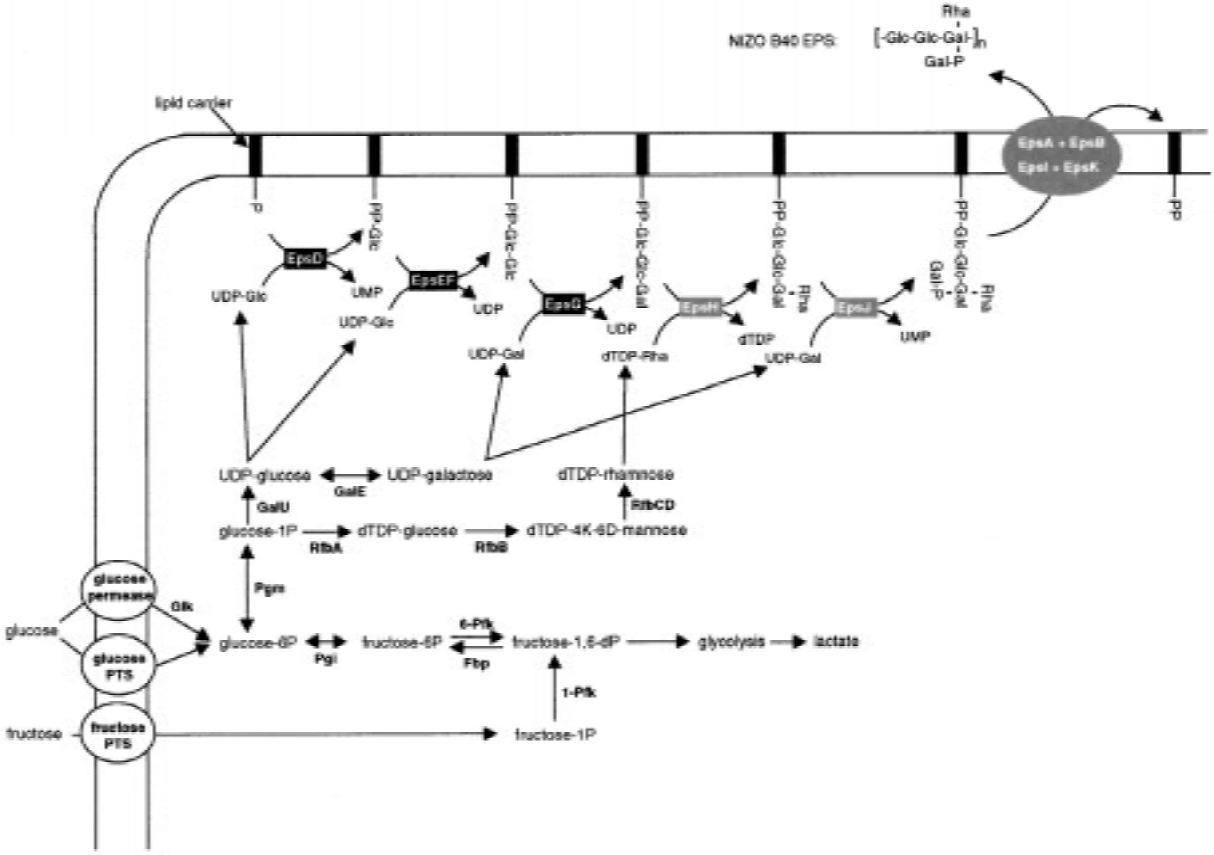

Figure 2. Schematic representation of pathways involved in NIZO B40 EPS biosynthesis. Both the general metabolism focussed on nucleotide-sugar biosynthesis from glucose or fructose as an external carbon source (lower left) and the *eps* genecluster encoded steps of repeating unit synthesis, export and polymerisation (upper part) are indicated. Enzymes involved in conversions in the general metabolism are indicated. The role of specific *eps* gene products has either been experimentally determined (black boxes) or is predicted by homologies (grey boxes). Glc, glucose; Gal, galactose; Rha, rhamnose. UDP-Glc, UDP-Gal and dTDP-Rha are nucleotide sugars.

identification of lipid-linked [^{14}C]-saccharides by thin layer chromatography and autoradiography. These experiments have shown that the biosynthesis of B40-polysaccharide backbone is initiated by the linkage of a glucose from UDP-glucose to the lipid carrier by priming-glucosyltransferase EpsD (van Kranenburg et al. 1997, 1999a). Subsequently, the addition of a second glucose from UDP-glucose to the lipid-linked glucose involves the combined activity of EpsE and EpsF, and finally, the addition of the third backbone-sugar moiety, galactose, from UDP-galactose to this carrier bound cellobiose involves the activity of EpsG (van Kranenburg et al. 1999a; Figure 1A, 2). The fourth step in repeating unit biosynthesis is expected to be the linkage of rhamnose from dTDP-rhamnose or galactosyl-phosphate from UDP-galactose to the galactose moiety of the lipid linked trisaccharide. The high unstability of dTDP-rhamnose prevents its use as substrate in the *in vitro* GTF assay, which could explain the lack of direct experimental data to support the suggested involvement of EpsH and EpsJ as rhamnosyltransferase and phosphogalactosyltransferase, respectively, in these later steps of B40 repeating unit biosynthesis (van Kranenburg et al. 1999a; Figure 2).

The diversity of EPSs produced by different *L. lactis* strains is illustrated by the variation of the chemical (sugar-) composition of the EPS produced and by the genetic organisation of the specific gene clusters involved (van Kranenburg et al. 1999b). Partial DNA sequence data of the *eps* gene clusters from *L. lactis* strains NIZO B891 and NIZO B35 revealed that both genetic organisation and sequence of the first genes, *epsRXABC*, are highly conserved (van Kranenburg et al. 1997, 1999b; Figure 1B). The organisation of the other known B891-*eps* genes (*epsDEF*) appears to be conserved relative to NIZO B40. The B35 *eps* cluster,

however, appears to be interrupted by an IS*982* element, which possibly indicates IS-element mediated rearrangement or horizontal gene transfer of parts of polysaccharide gene clusters. The *in vitro* functional analysis of the B891 and B35 priming GTF encoding genes (*epsD*-homologues) revealed that they encode a glucosyl- and galactosyl-priming transferase, respectively (van Kranenburg et al. 1999b; Figure 1B). Moreover, functional analysis of the B891 *epsDEF* genes (Figure 2B) led to a carrier-linked lactose, indicating that B891 EpsEF are involved in coupling of a galactose moiety from UDP-galactose to the carrier-bound glucose (van Kranenburg et al. 1999b).

A first and essential step towards EPS engineering was accomplished by creating a non-polar disruption mutation in the NIZO B40 *epsD* gene, which resulted in loss of EPS production. Interestingly, the *epsD* mutation could subsequently be complemented by introduction of a second plasmid encoding either the homologous NIZO B40 *epsD* gene, or the NIZO B891 *epsD* gene, both encoding a lactococcal priming glucosyltransferase (van Kranenburg et al. 1999b). Moreover, heterologous complementation of the *epsD* mutation was achieved by expression of the *S. pneumoniae* serotype 14 priming glucosyltransferase encoded by the *cps14E* gene, which is normally involved in CPS biosynthesis (Kolkman et al. 1996, 1997). These results are the first demonstration of functional complementation of a GTF mutation in a Gram-positive host by *in trans* expression of a gene encoding a similar activity. Moreover, they show that although transcriptional coupling of the genes involved is disturbed, the functional coupling of the enzymes encoded can apparently still occur. In contrast, expression of a priming galactosyltransferase derived from either *L. lactis* NIZO B35 (*epsD*) or from *S. thermophilus* Sfi6 (*epsE*; Stingele et al. 1996, 1998) did not result in complementation of EPS production (van Kranenburg et al. 1999b), suggesting an exclusive specificity for its natural substrate of one or more components of the B40-EPS biosynthesis machinery.

Recently, other examples that hint at the possibilities for production of heterologous or new EPS structures in *L. lactis* have been reported. Expression of the genes responsible for type 3 CPS production in *S. pneumoniae* resulted in high level production of the type 3 polysaccharide by *L. lactis* (see below; Gilbert et al. 1998). *L. lactis* cells in which the entire *S. thermophilus* Sfi6 *eps* gene cluster is expressed appeared to produce low but significant amounts of EPS, of which the structure differed from the EPS produced by the original *S. thermophilus* strain both in backbone composition (Gal instead of GalNAC) and sidechain substitution (lactococcal polymer lacks Gal sidechain). In contrast to the B40-epsD complementation studies (see above), this latter example illustrates that GTFs do not necessarily exhibit exclusive specificity for donor and acceptor sugar moieties and that the polymerase and export machinery in its turn might not be exclusively specific for one repeating unit (Stingele 1998).

These findings offer good perspective for future EPS engineering, although several bottlenecks caused by substrate specificity of the biosynthesis machinery will have to be overcome. Increasing knowledge on EPS and CPS structures, combined with the availability of individual biosynthetic components from *eps* and *cps* gene clusters will allow evaluation of the possibilities and limitations of genetic engineering strategies directed towards production of 'tailor made' EPS molecules in *L. lactis* or other LAB. Moreover, the role of specific *eps* gene products in repeating unit export, polymerisation and determination of polymer chain-length should be studied in more detail in order to manipulate these features. Especially chain-length engineering could generate EPSs of higher molecular weight, leading to better rheological properties (see below) or on the other hand could lead to the production of tailor made short-chain oligosaccharides.

Metabolic pathway engineering of EPS production

The industrial application of EPS produced by LAB is strongly hampered by the low production level, which is highly variable but ranges from 50 to 500 mg per liter. In the above section we have discussed the final steps of EPS biosynthesis that are mediated by the proteins encoded by the *eps* gene cluster. Besides these specific *eps* genes, EPS production also requires a number of so-called housekeeping genes that are involved in the biosynthesis of the EPS building blocks, the nucleotide sugars. Therefore, the physiology of nucleotide sugar biosynthesis in relation to EPS production levels should be evaluated to eventually use metabolic engineering as a means to increase EPS production by LAB. This approach should be supported by a kinetic model that describes the contribution of the individual enzymes to level of a complex end-product like EPS.

A possible key-step in nucleotide-sugar biosynthesis is the interconversion of glucose-6-phosphate,

an intermediate in sugar breakdown, and glucose-1-phosphate, the central precursor in nucleotide-sugar biosynthesis (Sjöberg & Hahn-Hägerdahl 1989), which is performed by the phosphoglucomutase PGM (Figure 2). *L. lactis* contains two distinct forms of PGM, one specific for β-glucose-1P (i.e. β-PGM) and the other apparently specific for α-glucose-1P (i.e. α-PGM) (Qian et al. 1994). Since the phosphorolysis of glucose, lactose and other carbon sources usually yields α-glucose-1P, α-PGM could be a key-enzyme in sugar-nucleotide biosynthesis. However, to our knowledge, only the gene encoding β-PGM has been cloned from *L. lactis* (Qian et al. 1997).

The central intermediate, glucose-1P, in its turn has to be converted to UDP-glucose, UDP-galactose and dTDP-rhamnose in order to fulfil the precursor-requirements for EPS biosynthesis by *L. lactis* NIZO B40 (Figure 2). Presently, all the genes that encode the enzymes that are putatively involved in the biosynthesis of these sugar-nucleotides from glucose-1P (*galU*, *galE*, and *rfbACBD*; Figure 2) have been cloned from *L. lactis* strain MG1363 (Grossiord et al. 1998a; Boels et al. 1998; Boels et al., unpublished results), thereby generating the tools to target specific endogenous enzymatic activities that could be bottlenecks in EPS biosynthesis, and thereby evaluate the potential of metabolic engineering strategies to improve EPS production levels.

The role of the Leloir enzyme galactose epimerase (GalE; Figure 2) was evaluated by analysis of NIZO B40 EPS biosynthesis in a *galE* mutant strain. EPS biosynthesis was abolished in *galE* mutant cells grown on media with glucose as a sole carbon source, but could be recovered by addition of galactose to the medium. These results indicate a crucial role for *galE* in the synthesis of UDP-galactose from glucose and thus in EPS biosynthesis by *L. lactis* (Boels et al. 1998). Moreover, the *galE* strain was also affected in cell division when grown on glucose alone, which probably reflects the critical role of UDP-galactose as a precursor in cell-wall biosynthesis (Grossiord et al. 1998b).

Gilbert et al. (1998) described the expression of the *cps3D* and *cps3S* genes from *S. pneumoniae* type 3 in *L. lactis*, leading to low heterologous production of type 3 polysaccharide. Interestingly, its production level could be strongly increased (to 15 μg per 10^8 cells) by co-expression of the *cps3U* gene that encodes a glucose-1-phosphate uridylyltransferase (GalU; Figure 2) analogue, indicating that the level of available UDP-glucose in lactococcal cells is limited by the endogenous GalU enzyme level. Functional overexpression of the lactococcal *galU* gene resulted in strongly increased levels of UDP-glucose and UDP-galactose in *L. lactis* (GalE maintains an almost stable ratio between the two nucleotide sugars; Figure 2), which confirms the hypothesis that the GalU enzyme activity level determines the intracellular levels of UDP-glucose and –galactose in wild-type cells (Boels et al., unpublished results). The effects of increased GalU levels on EPS production are currently being evaluated.

Rhamnose is an important residue in the O-antigen of lipopolysaccharides in many Gram-negative bacteria and the role of *rfb*-like genes in biosynthesis of the precursor dTDP-rhamnose is well established (Reeves 1993). Also in Gram-positive bacteria *rfb*-homologues appear to play an essential role in the production of rhamnose containing polysaccharides. The *cps19fL-O* genes are part of the *cps* gene cluster of *S. pneumoniae* type 19F and exhibit very strong homology with the *Shigella flexneri rfbA-D* genes and functionally complement a mutation deleting the respective *S. flexneri* homologues (Morona et al. 1997). An *S. pneumoniae cps19fL* mutant exhibited a so-called rough phenotype and had lost the capacity to produce CPS, confirming an essential role of the *rfb*-like genes in CPS-19F production (Morona et al. 1997). In *S. mutans*, the genes encoding the homologues of the *S. flexneri* RfbA, RfbB and RfbD proteins have been identified (designated *rmlA*, *rmlB* and *rmlC*, respectively) and functionally characterised. The gene encoding the RfbC homologue of *S. mutans* has not been identified sofar, but apparently does not map close to the *rml* locus (containing *rmlA-C*) on the *S. mutans* chromosomal map. In *S. mutans*, *rml* mutations led to a changed cell-wall composition (lacking rhamnose) and did no longer produce the serotype-specific polysaccharide antigen (Tsukioka et al. 1997). Functional analysis of the lactococcal *rfb* gene cluster (Boels et al. 1998) is especially interesting in terms of evaluation of EPS-engineering possibilities in *L. lactis*. Rhamnose is not a 'backbone-sugar' in NIZO B40 EPS, which is in contrast with the *S. pneumoniae* and *S. mutans* polysaccharides described above. Therefore, introduction of a plasmid carrying the NIZO B40-*eps* gene cluster in a *rfb* mutant strain might allow evaluation of side-chain specificity of the repeating-unit biosynthesis, export and polymerisation machinery. That this approach could be successful in creating a novel EPS with an altered structure, is supported

by the experiments described by Stingele (1998; see above).

Important indications of blockage points or rate limiting steps for EPS production by LAB could be obtained from studying the physiological conditions that influence the EPS production level in LAB. Evaluation of NIZO B40 EPS production levels under various physiological conditions showed that fructose grown cells produced much less EPS compared to glucose (or several other carbon sources) grown cells. This observation was also described for the level of EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Grobben et al. 1996). This fructose specific effect is possibly explained by the low activity level of fructose 1,6-bisphosphatase (Fbp; Figure 2) in *L. lactis* (Looijesteijn et al. unpublished results). This enzyme is required for conversion of fructose 1,6-dP to fructose-6-P, an essential step in generation of the central sugar nucleotide precursor glucose-1-P from glucose (via Pgi and Pgm; Figure 2). Preliminary results indicate that EPS production levels in fructose grown cells can be increased by functional overexpression of the heterologous *fbp* gene from *B. subtilis*, which confirms the assumed limitation of Fbp in *L. lactis*. Interestingly, also the growth rate of *L. lactis* on media containing fructose as sole carbon source was significantly increased by Fbp overexpression (Looijesteijn et al., unpublished results), suggesting that the sugar nucleotide requirements for efficient growth are also limited by the low Fbp activity in this organism. In analogy, the observed reduced growth rate of *L. lactis galE* mutant cells in media containing glucose as a sole carbon source, could be explained by limiting UDP-galactose levels (see also above).

Alternatively, the maximal EPS production level could in some cases be determined by the activity level of the factors encoded by the *eps* gene cluster, rather than by the level of sugar nucleotides. This possibility is supported by the finding that NIZO B40 EPS production can be increased by overexpression of *epsD* (van Kranenburg et al. 1999b). However, this hypothesis might not hold true for the biosynthesis of EPSs other than NIZO B40 EPS. The latter is supported by the findings that the level of *S. pneumoniae* type 3 CPS production in *L. lactis* can be dramatically increased by the expression of a pneumococcal GalU analogue (Gilbert et al. 1998).

Physical properties of EPS in watery solutions and products

A crucial factor in design of EPSs that would be desirable for a certain application is knowledge of the physical properties of different EPSs in solution and its interactions with other components of the product. Since in most cases, the desired contribution of EPS to the properties of a product is their thickening effect, it is important to understand the parameters that determine the viscosity of EPS solutions. Most EPSs can be described as random coil polymers that do not have a fixed shape but a randomly fluctuating tertiary structure. The relative viscosity η_r (with respect to the solvent) of EPS solutions is a function only of the intrinsic viscosity ($[\eta]$; a specific volume of the dispersed spherical particles in m^3/kg) and the concentration (c; g/l).

Recently, the following equation was derived, describing the master curve for random coil polysaccharide solutions (Tuinier et al. 1999a):

$$\eta_r = 1 + [\eta]c + 1/25\{[\eta]c\}^{3.5}$$

The differences between polysaccharides can thus be explained by differences in the intrinsic viscosity $[\eta]$. This quantity is proportional to the specific volume of the polysaccharide in solution and can be expressed as a function of the molar mass (M) and radius of gyration (R_g; a measure for the size of a polymer in solution). For random coil polysaccharides R_g depends on M ($R_g \sim M^{0.5-0.6}$) and for more expanded polysaccharides R_g is larger at a given M. These observations mean that, in order to obtain a higher (intrinsic) viscosity, either the molar mass (chain length) or the polysaccharide stiffness should be increased. Although the relation between polysaccharide chemistry and chain stiffness have not yet been established completely, some trends have been described (Rees 1977); (i) $\beta(1\rightarrow4)$ linkage of monosaccharides leads to a higher chain stiffness compared to $\beta(1\rightarrow2)$ or $\beta(1\rightarrow3)$ linkages (Lapasin & Pricl 1995), (ii) α linkages usually lead to more flexible chains than β linkages as is the case for $(1\rightarrow4)$ linkages (Lapasin & Pricl 1995) and (iii) branches and side groups influence the chain stiffness.

Static light scattering measurements of size-fractionated *L. lactis* NIZO-B40 EPS in aqueous solution at an ionic strength of 0.1 M generated a number-averaged molar mass of $1.47*10^6$ g/mol and radius of gyration of 86 $\pm$ 2 nm. Moreover, the relation of R_g and M for B40 EPS ($R_g \sim M^{0.57}$) is in good

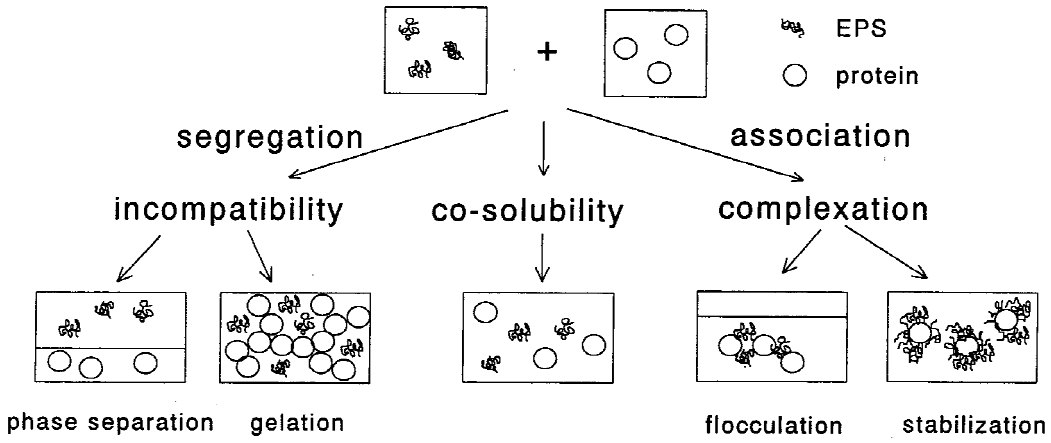

Figure 3. Schematic representation of the main trends in the behavior of protein/polysaccharide mixtures.

agreement with a random coil structure of this polysaccharide in solution (Tuinier et al. 1999b). Similar measurements with *L. lactis* NIZO B891 EPS showed that although the molar mass of this polysaccharide ($2.38^{*}10^{6}$ g/mol) is larger than that measured for B40 EPS, the radius of gyration of B891 EPS is only slightly larger (B891: Rg = 91± 2; $R_g \sim M^{0.51}$) (Tuinier 1999c). These observations suggest higher stiffness of the B40 EPS 'backbone' compared to B891 EPS. The β-(1-4) sugar linkages in the B40 EPS backbone support a relatively high stiffness of this polymer, which is also reflected in its radius of gyration and good rheological properties. However, since the primary structure of B891 EPS has not yet been fully determined one can only speculate about the reasons for the apparent higher flexibility of this polymer.

The developments described above, will in time allow a good prediction of the viscosity of a certain EPS molecule in solution. However, the contribution of EPSs to the properties of food products not only depends on the properties of the EPS itself but also on the interactions of the EPS with the various components in food products (e.g. proteins). When protein and polysaccharide solutions are mixed, the behaviour of the mixture depends on whether the two biopolymers are segregative (the biopolymers repel, are incompatible) or associative (the biopolymers attract, are compatible) (Tolstoguzov 1991). The main trends of mixing polysaccharides and proteins are (highly) schematically illustrated in Figure 3. For very dilute solutions the system is stable and proteins and polysaccharides are co-soluble. Upon increasing the concentration of the biopolymers the system may become unstable, depending on the type of interaction. As a rule proteins and polysaccharides tend to segregate (Tolstoguzov 1991), leading to a reduction of the polysaccharide concentration near the protein particle. This phenomenon is called depletion (Fleer et al. 1993) and exceeding a certain polymer concentration leads to a phase separation into protein-enriched and polysaccharide-enriched phases (Figure 3). These observations are illustrated by mixtures of B40 EPS with casein micelles, where a segregative interaction between the biopolymers takes place that leads to phase separation (Tuinier et al. 1999d). However, when the polysaccharides are very small compared to the protein particles and the volume fraction of particles is sufficiently high, aggregation of particles can lead to a space filling network, in a process called gelation (Syrbe et al. 1998).

A mixture of polysaccharides and proteins can also induce aggregation when associative interactions are operational (Syrbe et al. 1998) and the polysaccharides tend to adsorb onto the protein surfaces. If the amount of polymer is not sufficient to cover the protein surfaces, a polysaccharide may adsorb onto more than one protein surface, leading to (bridging) flocculation. In contrast, when the proteins are fully covered the polysaccharide-coated proteins are stabilised, which could be important if the proteins are initially unstable and the polysaccharide can thus act as stabiliser.

In fermented products EPS is produced during the growth phase of the EPS-producing LAB. In a product like yoghurt, EPSs play a significant role in the rheology of stirred yoghurt (van Marle 1998). Usually about 100 mg/l EPS is formed during yoghurt fermentation and the viscosity of stirred yoghurt depends very

strongly on the type of EPS produced. Also here, R_g and M appear to be the most important characteristics in determination of viscosity (Faber et al. 1998; Zoon et al., unpublished results).

Concluding remarks

Overall, one can conclude that the knowledge of EPS production in *Lactococcus lactis* (and other LAB) has developed impressively over the last years. The specific genetics and molecular biology involved in EPS production (*eps* genes) have been (partially) resolved for several EPS producing lactococci, and have reached a very exciting stage, where evaluation of possibilities and limitations of true genetic EPS-engineering can be envisaged. Moreover, the studies aiming for EPS production improvement have led to the identification and characterisation of interesting housekeeping genes. The encoded enzymes will be essential for design of metabolic engineering strategies to increase the EPS production level by LAB. However, until now, this approach has been hampered by the lack of predictive kinetic metabolic models that allow the identification and manipulation of the rate limiting steps of EPS biosynthesis by these organisms. The development of such models will add a clear rational to the metabolic engineering approaches.

Finally, the models developed to predict the behaviour and, consequently the effect of EPS addition to (food) products have already led to the identification of several important molecular properties (e.g. molar mass or chain length, polymer stiffness or monomer linkage-type in the EPS backbone) that determine their effects on a product property like viscosity. These models require further validation and fine-tuning to predict the exact properties of a specific EPS with for example a modification of the chemical composition or sugar-linkage-type in its repeating unit. Such predictive models will in the future allow the design of virtual EPSs that provide the desired product properties. A major challenge in the future for EPS- and metabolic-engineering will be the construction of modified lactococcal strains (or other LAB) that produce these 'designer' EPSs at a sufficiently high level.

Acknowledgements

Part of the work described here was supported by the European Community Biotechnology program (contracts BIOT-CT94-3055 and BIOT-CT96-0498). We thank Roland Siezen, Dick van den Berg and Oscar Kuipers for critically reading the manuscript.

References

Boels IC, Kleerebezem M, Hugenholtz J & de Vos WM (1998) Metabolic engineering of exopolysaccharide production in *Lactococcus lactis*. Proceedings 5th ASM on the genetics and molecular biology of streptococci, enterococci and lactococci, p. 66

Becker A, Niehaus K & Pühler A (1995) Low molecular-weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol. Microbiol. 16: 191–203

Faber EJ, Zoon P, Kamerling JP & Vliegenthart JFG (1998) The exopolysaccharide produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures. Carboh. Res. 310: 269–276

Fleer GJ, Cohen-Stuart MA, Scheutjens JMHM, Vincent B & Cosgrove T (1993) Polymers At Interfaces. Harper & Row

Gibson GR & Roberfroid MB (1995) Dietary modulation of the human colonic micobiota: introducing the concept of prebiotics. J. Nutr. 124: 1401–1412

Gilbert C, Robinson K, LePage RWF & Wells JM (1998) Heterologous biosynthesis of pneumococcal type 3 capsule in *Lactococcus lactis* and immunogenicity studies. Proceedings 5th ASM on the genetics and molecular biology of streptococci, enterococci and lactococci, p. 67

Grobben GJ, Smith MR, Sikkema J & de Bont JAM (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

Grossiord B, Vaughan EE, Luesink EJ & de Vos WM (1998a) Genetics of galactose-utilization via the Leloir pathway in lactic acid bacteria. le Lait 78: 77–84

Grossiord B (1998b) Metabolisme du galactose par la voie de Leloir l'operon gal de *Lactococcus lactis*. Thesis, l'École Nationale Supérieure Agronomique de Montpellier, Montpellier, France

Hosono A, Lee J, Ametani A, Natsume M, Hirayama M, Adachi T & Kaminogawa S (1997) Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotech. Biochem. 61: 312–316

Kitazawa H, Yamaguchi T, Miura M, Saito T & Itoh H (1993) B-cell mitogen produced by slime-forming, encapsulated *Lactococcus lactis* subspecies *cremoris* isolated from ropy sour milk, viili. J. Dairy. Sci. 76: 1514–1519

Kolkman MAB, Morrison DA, van der Zeijst BAM & Nuijten PJM (1996) The capsule polysaccharide synthesis locus of*S. pneumoniae* serotype 14: identification of the glycosyl transferase gene *cps14E*. J. Bacteriol. 178: 3736–3741

Kolkman MAB, van der Zeijst BAM & Nuijten PJM (1997) Functional analysis of glycosyltransferase encoded by capsular polysaccharide synthesis locus of *S. pneumoniae* serotype 14. J. Biol. Chem. 272: 19502–19508

Lamothe GT, Stingele F, Neeser JR & Mollet B (1998) Genetic analysis of exopolysaccharide (EPS) production in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Proceedings 5th ASM on the

genetics and molecular biology of streptococci, enterococci and lactococci, p. 67

Lapasin R & Pricl S (1995) Rheology of Industrial Polysaccharides: Theory and Applications. Blackie Academic Press

Liu D, Cole RA & Reeves PR (1996) An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. J. Bacteriol. 178: 2102–2107

MacPherson DF, Manning PA & Morona R (1995) Genetic analysis of the *rfbX* gene of *Shigella flexneri*. Gene 155: 9–17

Morona R, Mavris M, Fallarino A & Manning PA (1994) Characterization of the *rfc* region of *Shigella flexneri*. J. Bacteriol. 176: 733–747

Morona JK, Morona R & Paton JC (1997) Characterization of the locus encoding the *Streptococcus pneumoniae* type 19F capsular polysaccharide biosynthesis pathway. Mol. Microbiol. 23: 751–763

Nakajima H, Suzuki Y, Kaizu H & Hirota T (1992a) Cholesterol-lowering activity of ropy fermented milk. J. Food Sci. 57: 1327–1329

Nakajima H, Hirota T, Toba T, Itoh T & Adachi S (1992b) Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subspecies *cremoris* SBT0495. Carbohydr. Res. 224: 245–253

Qian N, Stanley GA, Hahn- Hägerdahl B & 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.

Qian N, Stanley GA, Bunte A & Rådström P (1997) Product formation and phosphoglucomutase activities in *Lactococcus lactis*: cloning and characterization of a novel phosphoglucomutase gene. Microbiology 143: 855–866

Rees DA (1977) Polysaccharide Shapes, Outline Studies in Biology. Chapman & Hall, London

Reeves P (1993) Evolution of *Salmonella* O-antigen variation by interspecific gene transfer on a large scale. Trends Genet. 9: 17–22

Sjöberg A & Hahn-Hägerdahl B (1989) ß-glucose-1-phosphate, a possible mediator for polysaccharide formation in maltose-assimilating *Lactococcus lactis*. Appl. Environ. Microbiol. 55: 1549–1554

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

Stingele F (1998) Exopolysaccharide production and engineering in dairy *Streptococcus* and *Lactococcus*. Proceedings 5th ASM on the genetics and molecular biology of streptococci, enterococci and lactococci, p. 31–32

Sutherland IW (1993) Microbial polysaccharides, In: Whistler RL & Miller JN Eds. Industrial Gums; polysaccharides and their derivatives, 3rd edition, (pp 69–85). Academic Press, San Diego

Sutherland IW (1998) Novel and established applications of microbial polysaccharides. TIBTECH 16: 41–46.

Syrbe A, Bauer WJ & Klostermeyer H (1998) Polymer science concepts in dairy systems. An overview of milk protein and food hydrocolloid interaction. Int. Dairy J. 8: 179–193.

Tolstoguzov VB (1991) Functional properties of food proteins and role of protein-polysaccharide interaction. Food Hydrocolloids4: 429–468.

Tsukioka Y, Yamashita Y, Oho T, Nakano Y & Koga T (1997) Biological function of the dTDP-rhamnose synthetic pathway in *Streptococcus mutans*. J. Bacteriol. 179: 1126–1134

Tuinier R, Zoon P, Cohen-Stuart MA, Fleer GJ & de Kruif CG (1999a) Concentration and shear-rate dependence of the viscosity of an exocellular polysaccharide. (Submitted for publication)

Tuinier R, Zoon P, Olieman C, Cohen-Stuart MA, Fleer GJ & de Kruif CG (1999b) Isolation and physical characterization of an exocellular polysaccharide. Biopolymers 49: 1–9

Tuinier R (1999c) An exocellular polysaccharide and its interactions with proteins. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands

Tuinier R & de Kruif CG (1999d) Phase behaviour of casein micelles/exocellular polysaccharide mixtures; experiment and theory. J. Chem. Phys. (In press)

van Kranenburg R, Marugg JD, van Swam II, Willem NJ, & de Vos WM (1997) Molecular characterization of plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. 24: 387–397

van Kranenburg R & de Vos WM (1998) Characterization of multiple regions involved in replication and mobilization of plasmid pNZ4000 coding for exopolysaccharide production in *Lactococcus lactis*. J. Bacteriol. 180: 5285–5290

van Kranenburg R, van Swam II, Marugg JD, Kleerebezem M & de Vos WM (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 HR, van Swam II, Kleerebezem M & de Vos WM (1999b) Functional analysis of glycosyltransferase genes from *Lactococcus* and other Gram-positive cocci: complementation, expression and diversity. (Submitted for publication)

van Marle ME (1998) Steady shear viscosity of stirred yoghurts with varying ropiness. Thesis, University of Twente, Enschede, The Netherlands

Vedamuthu ER & Neville JM (1986) Involvement of a plasmid in production of ropiness (mucoidness) in milk cultures by *Streptococcus cremoris* MS. Appl. Environ. Microbiol. 51: 677–682

Von Wright A & Tynkkynen S (1987) Construction of *Streptococcus lactis* ssp. *lactis* strains with a single plasmid associated with mucoid phenotype. Appl. Environ. Microbiol. 53: 1385–1386