Exopolysaccharides from lactic acid bacteria: perspectives and challenges

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Some lactic acid bacteria (LAB) secrete a polysaccharide polymer. This extracellular polysaccharide, or 'exopolysaccharide' (EPS), is economically important because it can impart functional effects to foods and confer beneficial health effects. LAB have a 'Generally Recognized As Safe' (GRAS) classification and are likely candidates for the production of functional EPSs. Current challenges are to improve the productivity of EPSs from LAB and to produce EPSs of a structure and size that impart the desired functionality. The engineering of improvements in these properties will depend on a deep understanding of the EPS biosynthetic metabolism and of how the structure of EPSs relates to a functional effect when incorporated into a food matrix.

Exopolysaccharides (EPSs) from lactic acid bacteria (LAB) have found their most valuable application in the improvement of the rheology, texture and 'mouthfeel' of fermented milk products, such as yoghurt. There is a high consumer demand for smooth and creamy yoghurt products, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatin). Consumer demand for products with low fat or sugar content and low levels of additives, as well as cost factors, make EPSs a viable alternative [1]. Although having no taste of their own, EPSs from LAB increase the time the milk product spends in the mouth, and hence impart an enhanced perception of taste [2]. An additional hypothesized physiological benefit is that EPSs will remain for longer in the gastrointestinal tract, thus enhancing colonization by probiotic bacteria [3]. In addition, LAB EPSs have been claimed to have antitumor effects [4], immunostimulatory activity [5,6] and to lower blood cholesterol [7].

EPSs are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. These sugar units are mainly glucose, galactose and rhamnose, in different ratios [8]. They are secreted into their surroundings during growth and are not attached permanently to the surface of the microbial cell [9]. This distinguishes them from the structurally similar capsular polysaccharides (CPSs), which do remain permanently attached to the surface of the cell. EPSs from microbial sources can be classified into two groups – homopolysaccharides (e.g. cellulose, dextran, mutan, alternan, pullulan, levan and curdlan) and heteropolysaccharides (e.g. gellan and xanthan) [9]. Homopolysaccharides consist of repeating units of only one type of monosaccharide (D-glucose or D-fructose) and can be divided into two major groups: glucans and fructans. By contrast, heteropolysaccharides from LAB have repeating units that demonstrate little structural similarity to one another [10]. The molecular mass of these polymers ranges between 4.0×10^4 and 6.0×10^6 Da [11]. The heteropolysaccharides are constructed from multiple copies of oligosaccharides [9], which contain between three and eight residues. Two or more different monosaccharides are usually present in each repeating unit and show different linkage patterns.

Structure–function relationships of EPSs

Because the polysaccharides derived from different LAB show large variation in composition, charge, spatial arrangement, rigidity and ability to interact with proteins, no defining correlation between EPS concentrations and viscosities has yet been established [2]. This correlation is particularly important because it will provide a foundation for a strategy aimed at producing functionally valuable polysaccharides, which will behave in a relatively predictable fashion when incorporated into food products. Some generalized trends have, however, become apparent: prerequisites for polymer solutions having high viscosity are high concentrations and also high specific volumes [12]. To achieve this, long chains of subunits (high molecular mass) and/or stiff chains are required [13]. Even though a relationship between chain stiffness and EPS composition has not yet been established, it is claimed there is evidence for this. Backbone linkages of the $\beta 1 \rightarrow 4)$ type, as found in *Lactococcus lactis* subsp. *cremoris* B40, for example, result in stiffer chains than $\alpha(1 \rightarrow 4)$ or $\beta(1 \rightarrow 3)$ linkages [12], and α-linkages result in more flexible chains than β-linkages [13]. Viscosity has also been correlated with increasing molecular mass in some instances [14].

Biosynthetic pathways leading to EPS synthesis in LAB

A key intermediate linking the anabolic pathways of EPS production and the catabolic pathways of sugar degradation appears to be glucose-6-phosphate, in which the flux of carbon bifurcates between the formation of fructose-6-phosphate toward the products of glycolysis, biomass and ATP formation and toward the biosynthesis of sugar nucleotides, the precursors of

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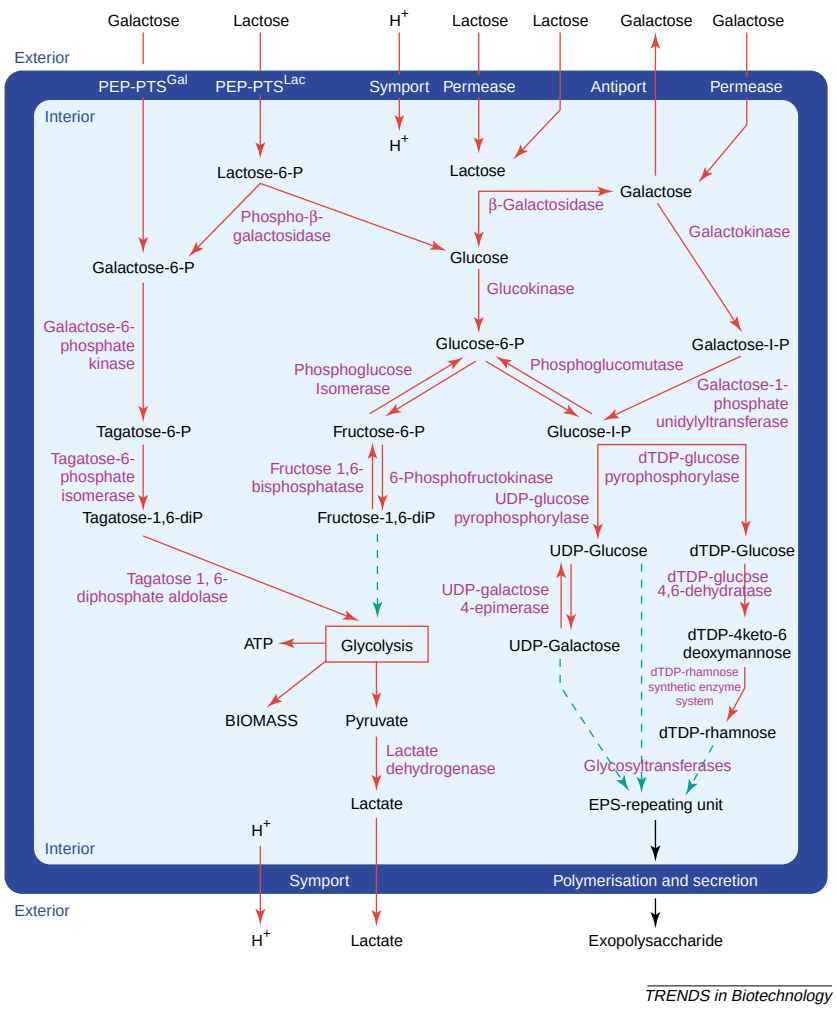

Fig. 1. Generalized diagram of the conversion of lactose, galactose and glucose to EPS and to glycolysis in lactic acid bacteria (glucose uptake not shown). In lactose-utilizing galactose-negative strains (e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus*), galactose is not metabolized and is expelled from the cell via a lactose/galactose antiport system.

EPSs (Fig. 1). Phosphoglutcomutase (PGM), the enzyme involved in the conversion of glucose-6-phosphate to glucose-1-phosphate, potentially has an important role in the divergence of flux between these catabolic and anabolic pathways [15,16]. Glucose-1-phosphate serves as a branch point for the formation of the sugar nucleotides UDP–glucose and dTDP–glucose via the action of UDP–glucose pyrophosphorylase and dTDP–glucose pyrophosphorylase, respectively. Note that these sugar nucleotides are used to form a variety of polysaccharides in the cell and hence the enzymes associated with their formation are shared (often termed 'housekeeping enzymes'). Conversion of galactose to glucose-1-phosphate via galactose-1-phosphate (the Leloir pathway) is possible if the system is present in the cell (Fig. 1). The subsequent stage of EPS synthesis in LAB – assembly of the monosaccharide repeating unit – is achieved by several EPS-specific enzymes, as identified initially in *Streptococcus thermophilus* S?6 [17] and in *L. lactis* NIZO B40 [18]. This repeating unit is assembled on a C55-isoprenoid–lipid carrier molecule, which is attached to the cytoplasmic membrane of the cell [19] (Fig. 2).

The sugars are linked to form the repeating unit by the action of several gene products on the EPS gene cluster (glycosyltransferases) [20]. The mechanism of polymerization of the repeating unit in LAB, and its subsequent export from the cell, is unclear. The high level of homology between Gram-positive and Gram-negative organisms with respect to the repeating unit synthesis means that it is likely that a similar mechanism will occur at the level of EPS polymerization and export. A simple model for this involves the action of a 'flippase' to move the lipid-bound repeating units from the cytoplasmic face of the membrane to the periplasmic face [9]. Using the same analogy, a polymerase could catalyse the linking of the repeating units and an enzyme could uncouple the lipid-bound polymer and control chain length.

Genetics of EPS production in LAB

The genes encoding EPS synthesis might be located in the plasmid, such as in *L. lactis* and *Lactobacillus casei* [18], or located on the chromosome, as in all the thermophilic LAB studied to the present time [10]. Stingele *et al.* [17] identified the *eps* genetic locus *of S. thermophilus* Sfi6, revealing a 15.25-kb region encoding 16 open reading

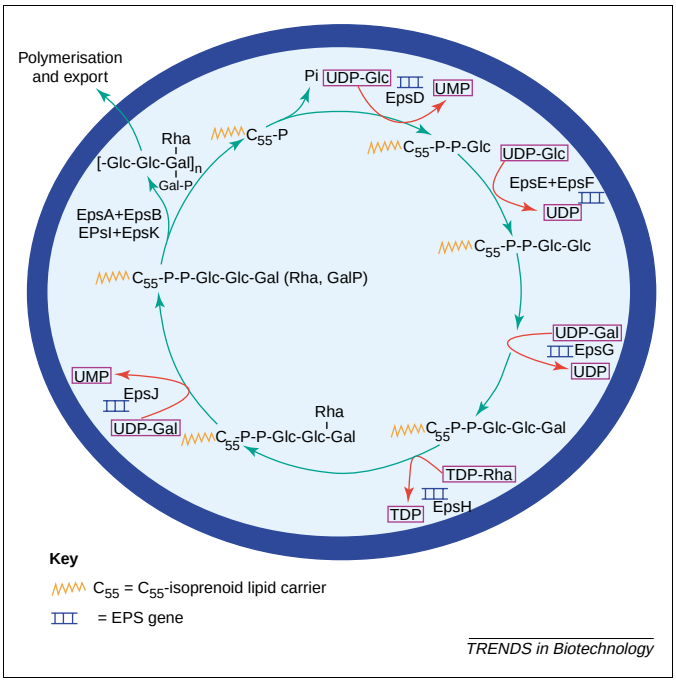

Fig. 2. Model of EPS biosynthesis in *Lactococcus lactis* NIZO B40. Adapted, with permission, from [39].

frames (ORFs), within which a 14.52-kb region encodes 13 genes (*epsA* to *epsM*) capable of directing EPS synthesis. Homology searches of the predicted proteins showed a high level of homology (40–68% identity) for *eps A, B, C, D* and *E* with the genes encoding CPS in *Streptococcus pneumoniae* and *Streptococcus agalactiae*. Van Kranenburg *et al.* [18] determined that all the essential information needed for the biosynthesis of EPS by *L. lactis* NIZO B40 was encoded in a single 12-kb gene cluster located on a single 40-kb plasmid (*epsRXABCDEFGHIJKL*), driven by a promoter upstream of *eps R* (Fig. 3). The predicted gene products of 11 of the 14 genes were homologous in sequence to gene products involved in EPS, CPS, lipopolysaccharide (LPS) or teichoic acid biosynthesis of other bacteria, and putative functions were assigned to these genes.

A high level of similarity between the gene clusters of other LAB is now becoming apparent: a recent study of the *eps* gene cluster of *Lactobacillus delbrueckii* subsp. *bulgaricus* revealed an 18-kb DNA region consisting of 14 genes (*eps A* to *eps N*), with a similar genetic

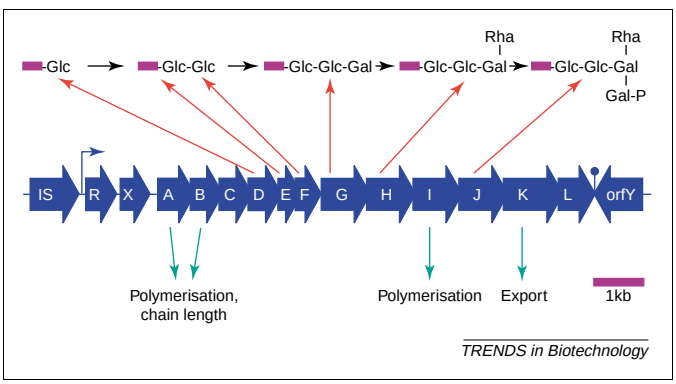

Fig. 3. Organization of the *eps* gene cluster in *Lactococcus lactis* NIZO B40. Adapted, with permission, from [18] (©Blackwell Science Ltd).

organization to other LAB [21]. The genes in the clusters are oriented in one direction and transcribed as a single mRNA [17,18,22]. The functional sequences of these clusters in Gram-positive bacteria, which synthesize polysaccharide at the cell surface, appear to follow a similar trend of regulation, chain-length determination, biosynthesis of the repeating unit, polymerization and export (Fig. 3) [22].

Strategies for enhancement of EPS production in LAB

One of the advantages of using LAB for metabolic engineering arises from the virtually complete uncoupling of their basic catabolic and cellular biosynthetic pathways [23]. There are no clear-cut fundamental principles for the metabolic engineering of LAB to overproduce or produce different EPSs. The broad range of structures and compositions of heteropolysaccharides produced by LAB, and their varying functional effects, means that there is no definitive way to establish a universal benchmark for desirable EPS titres. An economically viable titre of EPS produced in fermentation for use as a food additive has been quoted to be in the range of 10–15 g l^{-1} [24]. However, because of the cellular energy limitations of LAB, the highest EPS titre so far reported is 2.767 g l^{-1} in *Lactobacillus rhamnosus* RW-9595M [25]. In formulating a strategy for the overproduction of EPSs, it is useful to learn from past experiences of harnessing the genetic and metabolic capacity of these organisms to generate different (non-polysaccharide) products. Strategies that have been applied to achieve the rerouting of carbon flux to overproduce specific products include single, multiple and whole pathway engineering, the engineering of redox reactions and engineering global control systems [23].

A key consideration of LAB metabolism is the large proportion of carbon that flows to lactate; it has been suggested that if EPS production were coupled to growth of the cell then a reduction in the formation of lactate, which is known to inhibit growth, could elevate EPS formation [24]. More carbon could be diverted away from glycolysis and into EPS formation. This strategy has been used to obtain overproduction of metabolic end products such as alanine by overexpression of the *Bacillus sphaericus alaD* gene for an alanine dehydrogenase in an LDH-deficient strain of *L. lactis* [23]. It has also been effective for ethanol, acetoin, mannitol, 2,3-butanediol, succinate and pyruvate production by making a 'double knockout' strain of *Lactobacillus plantarum* (NCIMB 8826), in which the expression of L-LDH and D-LDH was attenuated [26]. If this strategy was adopted to achieve higher EPS yields, then it is anticipated that additional regulatory alterations would be necessary to ensure that excessive carbon was not diverted to unwanted metabolites via the pyruvate node. This approach of generating LDH-deficient or LDH-negative^{-}mutants is not without pitfalls and it is important to consider the impact it would have on the redox balance in the cell. Under anaerobic conditions, LAB transfer all reducing equivalents to the metabolic end-products and NAD^{+} is regenerated from NADH. In *Streptococcus mutans*, for example, abolition of LDH activity proved lethal because the organism cannot recycle NADH by alternative routes [15]. Restoration of this

balance could theoretically be achieved by exploiting an NADH oxidase (NOX), to create the NADH:NAD^+ ratio necessary to support the metabolic status needed for EPS overproduction. For example, overproduction of NADH oxidase was effected by cloning the *S. mutans nox-2* gene on the plasmid vector *pNZ*8020 into *L. lactis*, under control of the endogenous *nisA* promoter [27]. Similarly, NOX overexpression was achieved by cloning the *nox* gene under the control of the *nisA* promoter (NICE) in *L. lactis* [15,28].

In this instance, a significant reduction of lactate production implies a concomitant reduction in glycolytic flux and a negative effect on ATP formation. Because ATP is needed for the biosynthesis of sugar nucleotides and the C55-isoprenoid lipid carrier molecules, as well as being required for polymerization and export of the EPS, a reduction in available energy could be expected to compromise EPS formation. This view is supported by Looijesteijn *et al*. [14], who showed that the efficiency of EPS production in *L. lactis* was highest when ATP was in excess of the level required for cell growth.

The conversion of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase and the subsequent formation of UDP-glucose, which is catalysed by UDP–glucose pyrophosphorylase, have been proposed as potential controlling points in the production of EPS [15]. Overexpression of the *pgm* gene (for phosphoglucomutase) and the *galU* gene (for UDP–glucose pyrophosphorylase) result in an accumulation of UDP–glucose and UDP–galactose, respectively, in *L. lactis* [29]. More recently, overexpression of the *galU* gene, in combination with the *pgm* gene in *S. thermophilus* LY03 (Gal^-), was reported to have resulted in an increase in EPS yield from 0.17 to 0.31 g mol^{-1} carbon from lactose [30]. Interestingly, a Gal^+ mutant of *S. thermophilus* LY03 (TMB6010) was reported (in the same study) to generate even higher yields of EPS than the parent strain (0.24 g mol^{-1} carbon from lactose). The EPS yield was further raised to 0.36 g mol^{-1} of carbon when *pgmA* was knocked out. This raises the possibility of uncoupling the Leloir pathway from glycolysis: the glucose moiety of lactose can be used for glycolytic reactions and the galactose moiety for EPS formation [31]. It has been speculated that galactose could be used exclusively for cell function in galactose-using strains via the tagatose-6-phosphate pathway, and glucose could be used for EPS synthesis [8]. The Leloir enzyme, UDP-galactose-4-epimerase, could be a specific target for overexpression in some LAB; UDP-galactose has a crucial role in EPS formation in *L. casei* [32].

Another approach to enhancing the production of EPS exists at the level of biosynthesis of the EPS polymer, and in particular, by raising the activity of glycosyltransferases associated with this process. An example of this is the small increase in EPS production obtained because of overexpression of the priming glycosyl transferase *epsD* gene in *L. lactis* [19]. Similar overexpressions have been shown in *S. thermophilus, Lactobacillus helveticus* and *L. delbrueckii* subsp. *bulgaricus* [33].

An alternative method could involve cloning the entire *eps* gene cluster on a single plasmid with a high copy number, but strain stability in a production setting would remain a key consideration. Alternatively, plasmids containing the *eps* operon could be transferred into a different organism to generate raised levels of flux to EPS production as a consequence of higher endogenous metabolic flux. For example, heterologous expression of the complete *S. thermophilus* Sfi6 *eps* gene cluster into a strain of *L. lactis* (MG 1363) has been achieved [33]. The potential commercial value of using an approach of cloning entire operons or clusters of genes associated with EPS production is reflected by patent publications in this field [34,35]. The market application of such strains would, however, be subject to regulatory controls and public acceptance of these types of products.

Can EPS in LAB be structurally engineered?

Apart from the importance of raising the production of EPS in LAB, the EPS must impart the desired functional effect. Central to this goal is an understanding of the interaction between different EPS structures and components of a food matrix at the molecular level [1]. Molecular modelling has been used as a valuable tool in this regard. Faber *et al.* [36] described a method for constructing a conformational model of a heteropolysaccharide and applied it to the EPS produced by *L. helveticus* 766. Research into generating 'designer' polysaccharides from LAB is still in its infancy, and most of the work to date relating to the control of EPS structure in LAB has been in the area of glycosyltransferases. It has been proposed that information collected on the genes expressing these enzymes, coupled with information of donor- and acceptor-specificity could be used in a combinatorial fashion for the assembly of the biosynthetic mechanisms, which might generate polysaccharides that have desirable or novel structural attributes [37]. There is potential for controlling the formation of EPS structure by introducing new or existing glycosyltransferases into LAB [20]; gene shuffling with glycosyltransferases could also be a promising means of producing different linkages [1]. Additionally, manipulation of the functions of the genes involved in export, polymerization, and determination of chain length might present a means of altering EPS structure. The production of a desired EPS could also be achieved by control of the culture conditions [11].

In some instances, EPS structure has been found to depend on the carbon source. Structural analyses of the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture showed that the EPS consisted of repeating units of glucose and galactose (in the ratio 1:2.4) when grown on fructose; and of glucose, galactose and rhamnose in a ratio of 1:7.0:0.8 when grown on a mixture of fructose and glucose [38]. This approach might not be applicable to all LAB strains. For example, the EPS composition of *Lactobacillus sake* strain 0-1 is independent of the type of carbon source used [24].

Overview

The relatively inefficient conversion of energy from carbohydrates by LAB compared with aerobic producers of polysaccharides presents a technical barrier to economic production. The most valuable application from metabolic engineering of EPS production in LAB is most likely to

ensue from a combination of yield and structural enhancements, which impart valuable functional or health-promoting characteristics in designer-type foods. Thus far, no economically significant improvements in EPS production by LAB have been reported. Much more information is needed about the regulation of the EPS-synthesizing pathways before a significant impact will be seen on the titre and specific yield of microbial EPS production. An integrated approach requiring solutions to multiple metabolic constrictions in the metabolic pathways to EPS production will probably be necessary, requiring the combined skills of an experimental approach, *in silico* metabolic modelling, molecular modelling and combinatorial techniques. This effort will require the investigation of regulatory controls, energy availability, competition for precursors and substrates (e.g. the C55-isoprenoid lipid carrier), gene expression and physicochemical interactions. Because the energetics of LAB are such that the production of copious quantities of EPS are technologically unfeasible, successful exploitation of EPS from this group of bacteria will rely heavily on the specific functional effects of these molecules. The challenge will be to identify these 'actives', and then reverse-engineer or select LAB that can produce the desired polymers. Another approach could involve assembly of the desired polymer outside of the cell in a sequential fashion, using 'tailor-made' enzymes.

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