

Exopolysaccharides Produced from *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Abstract

Lactobacillus delbrueckii subsp. *bulgaricus*, which has been widely used as a fermented milk starter, is a type of probiotic, and certain strains are able to produce exopolysaccharide (EPS). EPS produced from *L. bulgaricus* contributes to the physical and biological function of dairy products by regulating immune response, and this tendency seems to place EPS with acidic groups. To date, six types of chemical structure have been determined and are basically composed from glucose (Glc), galactose (Gal), and rhamnose (Rha). *Eps* clusters on chromosome DNA control the EPS synthesis and are transcribed as one mRNA 14 genes with 18kb on *L. bulgaricus* Lfi5. Furthermore, *L. bulgaricus* is able to utilize lactose (Lac) as carbohydrate source, repeating units of EPS are synthesized from Glc 6-phosphate, generated by an Embden-Meyerhof (EM) pathway in cellular carbohydrate assimilation. This review discusses EPS produced from *L. bulgaricus*.

Keywords

Lactobacillus delbrueckii subsp. *bulgaricus*, Exopolysaccharide, Probiotics, EPS Synthesis, Carbohydrate Metabolism

1. Introduction

Lactobacillus delbrueckii subsp. *bulgaricus* is a facultative anaerobic gram-positive bacteria and an important starter for the production of fermented milk products such as yogurt and cheese. Yogurt is defined as “the fermented milk using both species of *Streptococcus thermophilus* and *L. bulgaricus*” in Codex Alimentarius. *L. bulgaricus* has been recognized as a probiotic that does not adhere well to the human intestine. Some strains of *L. bulgaricus* are able to produce exopolysaccharide (EPS) and its physiologic effects, such as enhancement/regulation of the immune response and cholesterol lowering activity, have been elucidated. In this paper, EPS pro-

duced from *L. bulgaricus* is discussed not only regarding industrial utilization but also its biochemistry, such as the metabolism of carbohydrates in bacterial cells.

2. Classification of EPS and Its Effects on the Properties of Fermented Milk

EPS can be divided into two groups according to the type of monosaccharide, that is, homo-polysaccharide or hetero-polysaccharide. Homo-polysaccharide is constituted from one type of monosaccharide, and hetero-polysaccharide is combined with several monosaccharides. Dextran produced from *Leuconostoc mesenteroides* represents as homo-polysaccharide. *L. mesenteroides* is able to secrete extracellular glucansucrase and synthesize α -glucan polymer from liberated glucose. Glucansucrase usually forms plural glycosidic linkages, and so dextran produced from *L. mesenteroides* contains not only α -(1-6) linkage but also 2,3,4-linkage [1]. Dextran, which contains various types of branching or molecular weight, can be isolated artificially and has been widely used in the medical and biochemical industries. Dextran derivatives have been developed and are widely used, leading to a high commercial value for dextran.

Hetero-polysaccharide, which consists of several monosaccharides, is synthesized by intracellular glycosyl-transferases [2]. Namely, saccharides taken into cells are utilized as an energy source by the Embden-Meyerhof (EM) pathway, and hetero-polysaccharide is synthesized from an intermediate of the glycolytic pathway in the cells. EPS produced from *L. bulgaricus* belongs to the hetero-polysaccharide group and acts as a thickener, stabilizer or gelling agent and prevents syneresis of dairy products. EPS interacts with milk proteins and has an effect on the viscosity and gelation of dairy products [3]. This effect is influenced by molecular weight, constituted monosaccharide of polysaccharide and/or the final pH of fermented milk [4].

3. Physiologic Effects of EPS

The physiologic effects of EPS produced from lactic acid bacteria have been demonstrated, and new findings have been reported. For instance, EPS produced from *Lactobacillus acidophilus* reduces allergies by affecting the pepsin/chymotrypsin hydrolysis of β -lactoglobulin [5]. EPS of *Lactobacillus johnsonii* from the intestinal tract of a healthy mouse had broad immune activity and affected the chemical structure [6]. Phosphorylated EPS from *Lactococcus lactis* subsp. *cremoris* resulted in anti-tumor and immune stimulation activity [7]-[9]. The cultures of *L. bulgaricus* were able to bind with higher amount of cholic acid than *S. thermophilus* [10], and there was a correlation between EPS production and cholesterol elimination [11]. Furthermore, in regard to EPS of *L. bulgaricus* OLL1073R-1, Ebina *et al.* demonstrated that EPS had host-mediated anti-tumor activity [12]. Subsequently, we found that this EPS consisted of two types of polysaccharides, neutral polysaccharide (NPS) and acidic polysaccharide (APS) [13], and the phosphate group in APS could enhance the immune response, such as in B-cell mitogenic activity [14] and cytotoxicity of the macrophage [15]. Makino *et al.* showed that APS or fermented milk by *L. bulgaricus* OLL1073R-1 could activate natural killer (NK) cells and decrease the incidence rate of colds or other influences [16]-[18].

4. Chemical Structures of EPS

The six repeating units of *L. bulgaricus* EPS are summarized in **Table 1** [19]-[25]. Although those orientations and binding sites have variety, EPS is mainly constituted from glucose (Glc), galactose (Gal), rhamnose (Rha) and branching chains. In rare cases, the furanose ring of Gal is included in one of the elucidated structures [19]. Based on this finding, it was determined that Gal (*f*) contributes to the conformation of EPS and has a random coil structure by analyzing the whole conformational space of the glycosidic bond and Φ and Ψ values of the glycosidic linkage.

Several studies have been conducted to determine the sugar composition of EPS, although chemical structure was not analyzed [13] [26]-[31]. In those studies, Glc, Gal and Rha were also found as the main substances, and other substances, such as mannose (Man), fructose (Fru) and proteins, may have been present. The sugar composition of *L. bulgaricus* NCFB2483 is the most complicated in the aforementioned research [29]. In this paper, *L. bulgaricus* NCFB2483 produced EPS, with a molecular weight of 2.5×10^5 Da, composed from Gal, Glc, Rha and Man with a ratio of 5:1:0.6:0.5, and a trace of glucosamine was also present. EPS produced from *L. bulgaricus* generally has a high ratio of Gal. The strain containing the highest ratio of Gal is NCFB2772 composed from Glc, Gal and Rha with a molar ratio of 1:5.7 - 6.3:0.8 [30]. *L. bulgaricus* CRL420 is only composed of Glc

Table 1. The chemical structures of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus*.

| Strain | The chemical structure of repeating unit | Reference |
|----------|--|-----------|
| LBB.B26 | $\begin{array}{c} \alpha\text{-D-Glcp} \\ \downarrow \textcircled{1} \\ \textcircled{6} \\ \rightarrow 3\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Galp}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Galp}\text{-}(1\rightarrow \end{array}$ | [19] [20] |
| LBB.B332 | $\rightarrow 3\text{-}\alpha\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rhap}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rhap}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow$ | [21] |
| NCFB2074 | $\begin{array}{ccc} \alpha\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glcp} & & \alpha\text{-D-Galp} \\ \downarrow \textcircled{1} & & \downarrow \textcircled{1} \\ \textcircled{3} & & \textcircled{6} \\ \rightarrow 4\text{-}\alpha\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow & & \\ \downarrow \textcircled{2} & & \\ \beta\text{-D-Galp}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glcp} & & \end{array}$ | [22] |
| EU23 | $\begin{array}{c} \alpha\text{-L-Rhap} \\ \downarrow \textcircled{1} \\ \textcircled{3} \\ \rightarrow 2\text{-}\alpha\text{-L-Rhap}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rhap}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glcp}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glcp}\text{-}(1\rightarrow \end{array}$ | [23] |
| rr, Lfi5 | $\begin{array}{ccc} \beta\text{-D-Galp} & & \beta\text{-D-Galp} & & \alpha\text{-L-Rhap} \\ \downarrow \textcircled{1} & & \downarrow \textcircled{1} & & \downarrow \textcircled{1} \\ \textcircled{3} & & \textcircled{4} & & \textcircled{3} \\ \rightarrow 2\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glcp}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Galp}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Galp}\text{-}(1\rightarrow \end{array}$ | [24] [34] |
| 291 | $\begin{array}{c} \beta\text{-D-Galp}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glcp} \\ \downarrow \textcircled{1} \\ \textcircled{6} \\ \rightarrow 4\text{-}\beta\text{-D-Glcp}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glcp}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Galp}\text{-}(1\rightarrow \end{array}$ | [25] |

and Fru and does not include Gal [26]. In our previous study, it was shown that EPS produced from *L. bulgaricus* OLL 1073R-1 was composed of Gal and Glc, with a ratio of 3:2 [13].

In addition, acid functional groups may occasionally be present in EPS. In our previous study, a phosphate group was included in APS from *L. bulgaricus* OLL1073R-1 [13]-[15] [32]. Based on the ratio of phosphorus, phosphate groups are likely to occur at random intervals but may not invariably contain repeating units. The binding position of phosphate is also unidentified. It is very important for industrial development to investigate the introduction of phosphorus in detail, because the physiological function of EPS is improved by the introduction of the phosphate groups. It is expected that this will be elucidated in the future.

5. Genes of EPS

The EPS synthesis genes of *L. bulgaricus* are encoded on chromosome DNA and transmitted horizontally from *S. thermophilus* during utilization as starters of fermented milk with *S. thermophilus* strains [33]. In this study, however, some *eps* genes have been shown with a complete genome analysis, and there is only one paper that discusses the *eps* cluster on *L. bulgaricus* [34]. In this paper, it was demonstrated that *eps*DNA in *L. bulgaricus* Lfi5 encoded 14 genes (*epsA* to *epsN*) with 18 kb, and the *eps* cluster was transcribed as one mRNA. Its sequence was very similar to that of *S. thermophilus*. Genes encoding glycosyltransferases for biosynthesis of EPS were the same as the number of monosaccharides in repeating units, and the stereospecificity of each GTF gene was also identified. Based on this finding, it appears that it is necessary to increase the production to suppress regulation and enhance polymerization on *eps* gene expression.

To date, the complete genome of *L. bulgaricus* has been demonstrated on ATCC11842, ATCC BAA365 and ND02 [35]-[37]. ND02, which is able to produce EPS, was very similar to ATCC11842 and BAA365 on EPS genes, although a polysaccharide synthesis protein and an *eps* gene cluster were contained in several unique

gene clusters [37]. Furthermore, a portion of the sequences of *epsB* (536bp; Accession No. DQ087529) and *epsC* (596bp; Accession No. DQ087530) of *L. bulgaricus* rr were shown [38]. This paper also demonstrates that the glycosyltransferase was encoded by *epsE*.

6. Carbohydrate Metabolism and EPS Synthesis of *L. bulgaricus*

L. bulgaricus uses lactose (Lac) as an energy source and performs homo-fermentation, which generates lactic acid as a final fermentation product; ethanol may be produced depending on the condition of the culture. *L. bulgaricus* can assimilate Lac not just Glc, which results in an adaptation of milk. Simultaneously, this species is able to assimilate Man and Fru, whereas Gal can't be metabolized because it lacks a Leloir pathway. *L. bulgaricus* generally intakes Lac in to the cells by lactose permease on the cell membrane, and Gal is produced by Lac hydration of intracellular β -galactosidase, which is exhausted outside a cell. Welman *et al.* suggested that Gal exhausts to the outside of the cells via a Lac/Gal antiport system on *L. bulgaricus* [39] [40]. They also suggested that EPS is synthesized by sugar precursors, which consist of a combination of Glc 1-phosphate and sugar nucleotides such as UDP or dTDP.

In 2002, Lamothe *et al.* showed intracellular synthesis of EPS on *L. bulgaricus* [34]. Based on their report, repeating units of EPS are synthesized by EpsE (phospho-GTF), EpsF ($\alpha(1,3)$ GalT), EpsG ($\alpha(1,3)$ RhaT), EpsH ($\beta(1,3)$ GalT), EpsI ($\alpha(1,2)$ GalT), EpsJ ($\beta(1,4)$ GalT) and EpsM ($\beta(1,4)$ GalT). EpsB, EpsC and EpsD determine polymerization and chain length. It has been confirmed that EPS synthesis in this strain is glycosylated by UDP-Glc, UDP-Gal and dTDP-Rha as sugar donors. And repeating units polymerize and exhaust to the outside of a cell by EpsK and EpsN, respectively.

L. bulgaricus has been used as a starter with *S. thermophilus* in fermented products, and these species are symbiosis. Concerning symbiosis of these species and EPS, co-cultivation with *S. thermophilus* promotes EPS production of *L. bulgaricus*, whereas EPS produced from *L. bulgaricus* does not influence the growth of *S. thermophilus* [41] [42]. In addition, it has been demonstrated that *L. bulgaricus* produces EPS in a stationary phase [42]. Based on these findings, it may be suggested that bacterial cells accelerate EPS production. To date, there has been no report regarding EPS synthesis and cell contact or quorum-sensing peptides on *L. bulgaricus* [41] [42]; I expect to elucidate these relationships in future.

7. Conclusion

Recently, we reported on formic acid, morphological change and EPS synthesis on *L. bulgaricus* OLL1073R-1 [43]. EPS production and its effect on *L. bulgaricus* are still unknown. It is very important for the development of the dairy industry to investigate the properties of lactic acid bacteria, particularly the influence and biochemical role of EPS produced from probiotics against intestinal microflora [44] [45]. More studies regarding the effects of EPS on lactic acid bacteria will be conducted in the future.

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