Note

Production of Exopolysaccharide by *Lactobacillus plantarum* and the Prebiotic Activity of the Exopolysaccharide

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Characteristics of an exopolysaccharide (EPS) produced by a mutant strain of *Lactobacillus plantarum*, strain 301102S, including yield from whey, monosaccharide analysis, and prebiotic activity, were investigated. The EPS production in whey was higher at 25°C than at 30 and 37°C. The supplementation with yeast extract and glucose in whey gave a high EPS yield of 145 mg/L, and the EPS contained glucose and mannose (molar ratio, 1:2). Prebiotic activities of galactooligosaccharide, inulin and the EPS with 37 lactic acid bacteria strains were investigated, and prebiotic activity of the EPS was high with the parent strain. This suggests that the EPS produced in whey by the mutant strain is easier utilized by strain 301102 than the other strains, and the EPS produced by this strain is capable for use as a prebiotic.

Keywords: exopolysaccharide, prebiotics, synbiotics, *Lactobacillus plantarum*

Introduction

Lactic acid bacteria (LAB) are used in many fermented foods particularly fermented dairy products such as cheese, buttermilk, and fermented milk. LAB produce lactic acid, carbon dioxide and diacetyl/acetoin that contribute to the flavor, texture, and shelf life of fermented foods. Fuller (1989) was the first to propose the term “probiotic,” and recently, its definition was further refined to “Live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host” (FAO/WHO, 2002). Probiotic LAB are a representative of live food ingredients that exert a beneficial effect on the health of the host. Probiotic numbers are enhanced by prebiotics, which are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacterial species already resident in the colon, and thus improving host health” (Gibson and Roberfroid, 1995). The term symbiotic is used when a product contains both probiotics and prebiotics.

Many studies have suggested that consumption of symbiotic products has greater beneficial effects on human health than probiotic or prebiotic products (Gopal *et al.*, 2001; Kekkonen *et al.*, 2007). Several studies have shown that the abilities of lactobacilli and bifidobacteria to ferment prebiotic carbohydrates are both strain and substrate specific (Kaplan and Hutkins, 2003; Huebner *et al.*, 2007). It is important to understand which prebiotics are metabolized by specific strains of bacteria, especially for those organisms whose intended use are as probiotics.

Most of the current prebiotics are low molecular weight except for inulin. Long carbohydrate chains will be metabolized more slowly than short ones, and polysaccharides thus exert prebiotic effects in more distal colonic regions than oligosaccharides, which are more rapidly fermented in the proximal colon (Rastall, 2003). Generally, exopolysaccharides (EPSs) play a major role as natural texturizers in the industrial production of yogurts, cheeses, and milk-based desserts. EPSs produced by LAB have received increasing attention mainly because of their health benefits. Immune stimulation, antimutagenicity, and antitumor activity of fermented dairy products prepared with EPS-producing LAB or EPSs themselves have been investigated (Chabot *et al.*, 2001; Kitazawa *et al.*, 1998; Sreekmar and Hosono, 1998; Tsuda *et al.*, 2008b). However, studies of applications using EPSs produced by LAB as prebiotics are limited. In general,
it is very unlikely for bacteria to use an EPS as energy, but it was reported that some LAB strains can use polysaccharides from Lactobacillus (Ruijssenaars et al., 2000; Korakli et al., 2002). EPSs produced by LAB are possible to use as prebiotics and the use of an EPS-producing LAB strain as a starter may contribute to the development of synbiotic products.

The object of this study was a primary evaluation of the prebiotic effect of an EPS produced by Lb. plantarum against various LAB strains.

In the present study, whey and whey-based media were used for the LAB production of the EPS. Synthetic and semisynthetic media, such as MRS medium, are expensive and may not be suitable for food additives. In industrial applications, large quantities of whey are produced when making cheese. The relatively low cost of a potential media like whey makes the efforts well worth trying.

Lactobacillus plantarum 301102 identified from biological characteristics and 16S rDNA sequence was isolated from traditional home-made cheese from Inner Mongolia, China. Survivability and proliferation of this strain in porcine gastrointestinal tract following oral administration have been reported (Tsuda et al., 2007; Tsuda et al., 2008a). The authors have also reported that the EPS produced by the mutant strain 301102S, which was obtained from strain 301102 by exposure to mutagenic action of acridine orange and novobiocin, has antimutagenic action against heterocyclic amines, such as Trp-P-1 (Tsuda et al., 2008b). In the present paper, the characteristics of the EPS produced in whey, including yield, monosaccharide composition and prebiotic activity, are presented.

### Materials and Methods

**Bacterial strain** The 37 LAB and one Escherichia coli strains used in the present study are shown in Table 1. The strains were isolated at our laboratory unless otherwise stated. LAB strains were incubated in TYG broth (tryptone 10 g/L, yeast extract 5.0 g/L, glucose 5.0 g/L, Tween 80 1.0 g/L, and L-cysteine HCl monohydrate 0.1 g/L, pH 6.8 ± 0.2), E. coli was incubated in LB broth (Nacalai Tesque, Kyoto, Japan), and stocked in 10% reconstituted skim milk at -20°C. An inoculum of 1% was used in all tests.

**Effects of incubation condition on exopolysaccharide production** EPS production by Lb. plantarum 301102S was assessed as follows (Christiansen et al., 2001). Reconstituted sweet whey (Snow Brand, Tokyo, Japan) was used at a concentration of 13% (w/v), which in preliminary experiments proved to be optimal for slime formation. The whey was heated in a centrifuge bottle to 70°C for 10 min and cooled in a water bath. The precipitate was removed from the whey by centrifugation at 1000 × g for 10 min, and then 5 mL of the whey was dispensed to graduated Spitz tubes and inoculated with strain 301102S aseptically and incubated. The fermented whey was centrifuged at 1000 × g for 20 min, and the volume of precipitated slime was measured. The precipitated slime, for which yield was expressed as mL/5mL whey, consisted of EPSs, proteins, salts and bacterial cells.

### Table 1. Strains used in the present study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Species</th>
<th>Strain No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus thermophilus</td>
<td>16230</td>
<td>Lactobacillus casei</td>
<td>NILGS L-14</td>
</tr>
<tr>
<td></td>
<td>18235</td>
<td></td>
<td>L-49</td>
</tr>
<tr>
<td></td>
<td>2330M2</td>
<td></td>
<td>34143</td>
</tr>
<tr>
<td></td>
<td>3011M4</td>
<td></td>
<td>34143S</td>
</tr>
<tr>
<td>Streptococcus. bovis</td>
<td>J2 40-2</td>
<td></td>
<td>ATCC* 393</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis</td>
<td>KM</td>
<td>Lactobacillus paracasei</td>
<td>ATCC 25598</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis</td>
<td>NILGS* N-7</td>
<td>Lactobacillus plantarum</td>
<td>IFO 3070</td>
</tr>
<tr>
<td></td>
<td>NILGS 527</td>
<td></td>
<td>6214</td>
</tr>
<tr>
<td></td>
<td>IFO* 12007</td>
<td></td>
<td>301102</td>
</tr>
<tr>
<td></td>
<td>DHI</td>
<td></td>
<td>301102S</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>TH15</td>
<td>Lactobacillus delbrueckii subsp.</td>
<td>ATCC 9649</td>
</tr>
<tr>
<td></td>
<td>RIMD* 3116001</td>
<td>delbrueckii subsp. lactis</td>
<td>1135</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>C210</td>
<td>Lactobacillus delbrueckii subsp.</td>
<td>306701</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>OR-1</td>
<td>bulgaricus</td>
<td>7235</td>
</tr>
<tr>
<td>Pediococcus acidilactici</td>
<td>ID-7</td>
<td>Lactobacillus acidiophilus</td>
<td>NILGS L-54</td>
</tr>
<tr>
<td></td>
<td>JCM* 5885</td>
<td></td>
<td>305501</td>
</tr>
<tr>
<td>Pediococcus dextrinicus</td>
<td>JCM 5887</td>
<td></td>
<td>306704</td>
</tr>
<tr>
<td>Pediococcus pentosaceus</td>
<td>JCM 5890</td>
<td></td>
<td>D6404</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>RB</td>
<td>Lactobacillus helveticus</td>
<td>130B4</td>
</tr>
</tbody>
</table>

*: NILGS: National Institute of Livestock and Grassland Science; RIMD: Research Institute for Microbial Diseases; JCM: Japan Collection of Microorganisms; ATCC: American Type Culture Collection; IFO: Institute for Fermentation, Osaka.
The consistency of the slime was evaluated organoleptically first with a loop. Incubation temperatures of 25, 30 and 37°C were applied to investigate the effect of incubation temperature on EPS production. Yeast extract, soy peptide (hinute, Fujisefyuyu), tryptone, peptone and Lab-remco powder were supplemented in the whey as a nitrogen source at 0.3% (w/v), respectively, and glucose, galactose, sucrose, maltose, raffinose and fructose were supplemented in the whey as a carbon source at 0.5% (w/v), respectively.

All assays were performed at least three times.

Preparation of exopolysaccharide  The modified method of Lindsay et al. (2003) was used to prepare the EPS from fermented whey. *Lb. plantarum* 301102S was cultured in 13% reconstituted whey at 25°C for 18 h. An 80% (w/v) trichloroacetic acid (TCA) solution was added to provide a final concentration of 14% TCA. The resulting mixture was centrifuged at 10,000 x g for 30 min at 4°C to remove cells and protein. The EPS in the supernatant was precipitated with two volumes of cold ethanol, followed by incubation for 4 h at 8°C. After centrifugation at 6000 x g for 10 min, the precipitation of the EPS was dissolved in deionized water. The EPS was precipitated with ethanol again, and subsequently lyophilized.

The lyophilized EPS was analyzed for carbohydrate and protein content and molecular weight distribution. The total amount of carbohydrate in the lyophilized EPS was determined with the phenol-sulphuric acid method using glucose as the standard (Dubois et al., 1956). Protein content was determined with the protein-dye binding method using bovine serum albumin as the standard (Bradford, 1976). Molecular weight distribution was investigated with gel permeation chromatography (column: Sephacryl S-200; mobile phase: 50 mM NH₄HNO₃; flow rate: 0.4 mL/min, and fractions were collected at 2.5-min intervals and the carbohydrate content of fractions was analyzed with the phenol-sulphuric acid method.

All assays were performed at least three times.

Monosaccharide analysis of exopolysaccharide Mono-saccharide analysis was conducted as follows (Calsteren et al., 2002). The lyophilized EPS was hydrolysed in 2 M trifluoroacetic acid at 120°C for 2 h. The sugar composition was determined by HPLC with refractive-index detection (column: NH2P-50 4E, Shodex; mobile phase: 80% acetoni-trile; flow rate: 0.8 mL/min; temperature: 30°C). Glucose, galactose, fucose, rhamnose, mannose, arabinose and xylose were used as the standards.

Prebiotic activity of exopolysaccharide Prebiotic activities of Oligomate HP (Yakult, Minatoku, Japan) as GOS, inulin and EPS against glucose were tested with 37 LAB strains. Oligomate HP contained more than 50% GOS, and less than 50% monosaccharide and lactose. Prebiotic activities were determined by the method of Huebner et al. (2007, 2008). Briefly, LAB and *E. coli* strains were inoculated into TY broth (tryptone 10 g/L, yeast extract 5.0 g/L, Tween 80 1.0 g/L, and L-cysteine HCl monohydrate 0.1 g/L, pH 6.8 ± 0.2) containing 0.2% (w/v) glucose, GOS (Oligomate HP), inulin and the lyophilized EPS, respectively, and incubated for 18 h at 37°C in consideration of the digestive tract environment *in vivo*. Optical density at 660 nm (OD660) of the culture was measured at 0 and 18 h. All assays were performed at least five times. Prebiotic activity was determined using the following equation.

\[
\text{Growth rate} = \frac{(\text{Log OD660 of TYP at 18 h} - \text{Log OD660 of TYP at 0 h})}{(\text{Log OD660 of TYG at 18 h} - \text{Log OD660 of TYG at 0 h})}
\]

Prebiotic activity = growth rate of LAB – growth rate of *E. coli*

TYP: TY broth containing prebiotics (GOS, inulin and EPS), respectively
TYG: TY broth containing glucose

Results and Discussion

Effects of incubation condition on exopolysaccharide production The effect of incubation temperature on EPS production in strain 301102S was investigated (Fig. 1A). The highest yield of slime was attained at 25°C, which was lower than the optimal growth temperature of 30°C for strain 301102S. Therefore, an incubation temperature of 25°C was applied in the series of tests. The effect of supplementation with a nitrogen source in whey was investigated (Fig. 1B), and the maximum yield was gained when yeast extract was supplemented. The effect of supplementation with a carbon source was also investigated (Fig. 1C), and the maximum yield was gained at 24-h incubation with sucrose or maltose supplementation and high yield was gained after 24-h incubation with glucose supplementation. From these results, yeast extract and glucose, sucrose and maltose were used to investigate the effects of supplementation with a nitrogen source and carbon source in combination. In this test, the higher yield was gained when yeast extract and glucose were supplemented than when yeast extract and sucrose or maltose were supplemented (data not shown).

There have been some reports that higher yield was gained with temperatures lower than the growth temperature with mesophilic LAB (Tallon et al., 2003; Degeest et al., 2001a, b) An unsuitable condition for growth is thought to be an optimal condition for EPS production by mesophilic LAB since sugar nucleotides, which are utilized by the cell wall, are needed for EPS production.

The yields reduced after reaching a maximum, as many
studies have reported, and this is caused by enzymes, such as glycohydrolase, produced by bacteria (Pham et al., 2000).

**Characteristics of exopolysaccharide** The EPS from 13% reconstituted whey supplemented with yeast extract and glucose after fermentation at 25°C for 18 h was purified with ethanol precipitation and the EPS yield was 145 mg/L. On the other hand, the EPS yield from simple whey after fermentation at 25°C for 96 h was 74 mg/L. That lyophilized EPS produced by strain 301102S contained 81% carbohydrate and 1.6% protein. This lyophilized EPS was eluted from the void-volume on the Sephacryl S-200. Therefore, this lyophilized EPS contains polysaccharides with molecular weights larger than $8 \times 10^4$. Monosaccharide analysis of this EPS was done using seven monosaccharides (glucose, galactose, fucose, rhamnose, mannose, arabinose and xylose) as standards, which are generally known as constituents of EPSs, and glucose and mannose were in this lyophilized EPS (molar ratio, 1:2) (Table 2). Glucose, galactose and rhamnose are usual components of many EPSs produced by LAB. Galactose and rhamnose, however, were not involved in the EPS production by strain 301102S; glucose and mannose were involved. There are no data in the literature regarding metabolic routes that could explain the incorporation of mannose units in EPSs produced by LAB, although the presence of this sugar has been already described (Sanchez et al., 2006; Knoshaug et al., 2000; Aslim et al., 2006). Some authors suggest that the detection of sugars such as mannose, arabinose, or xylose can be attributed to contamination from material coming from medium components such as yeast extract (Sanchez et al., 2006). Monosaccharide analysis of the EPS in simple 13% whey showed the presence of mannose.

This result may confirm that mannose was a component of this EPS.

The quantities of EPSs produced in milk by lactobacilli vary greatly. Quantities of EPSs were 50-60 mg/L for *Lactobacillus casei*, 60-150 mg/L for *Lactobacillus bulgaricus* (Cerning, 1995), and one of the highest yields was 2775 mg/L from whey permeate supplemented with yeast extract, vitamins, salts and amino acids for *Lactobacillus rhamnosus* RW-9595M (Macedo et al., 2002). The yield of the EPS for strain 301102S was 145 mg/L in whey supplemented with yeast extract and glucose. The present study suggests that whey, a low-cost safe media, supports production of EPSs by *Lb. plantarum* 301102S.

**Prebiotic activity of exopolysaccharide** Prebiotic activities of prebiotic sugar against glucose with 37 LAB strains were investigated, and strains showing positive prebiotic activity are listed in Table 3. *Pediococcus acidilactici* ID-7

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### Table 2. Relative monosaccharide compositions of the EPS produced by *Lactobacillus plantarum* 301102S in 13% whey supplemented with yeast extract and glucose after 18 h of incubation at 25°C.

<table>
<thead>
<tr>
<th>Yield (mg/L)</th>
<th>Arabinose</th>
<th>Fucose</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Rhamnose</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS in whey</td>
<td>74 (6.2)</td>
<td>ND</td>
<td>ND</td>
<td>1.0</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EPS in supplemented whey</td>
<td>145 (10.3)</td>
<td>ND</td>
<td>ND</td>
<td>1.0</td>
<td>2.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviations (n=3). ND: not detected.
Table 3. Prebiotic activity scores of galactooligosaccharide (GOS), inulin and the EPS produced by strain 301102S.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>GOS</th>
<th>Inulin</th>
<th>EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3116001</td>
<td>0.03</td>
<td>(0.02)</td>
<td>-0.01</td>
</tr>
<tr>
<td>ID-7</td>
<td>0.01</td>
<td>(0.01)</td>
<td>0.05</td>
</tr>
<tr>
<td>5885</td>
<td>-0.07</td>
<td>(0.01)</td>
<td>0.01</td>
</tr>
<tr>
<td>L-49</td>
<td>-0.08</td>
<td>(0.06)</td>
<td>-0.13</td>
</tr>
<tr>
<td>3070</td>
<td>0.00</td>
<td>(0.02)</td>
<td>0.01</td>
</tr>
<tr>
<td>301102</td>
<td>0.01</td>
<td>(0.00)</td>
<td>0.01</td>
</tr>
<tr>
<td>301102S</td>
<td>0.01</td>
<td>(0.01)</td>
<td>0.01</td>
</tr>
<tr>
<td>306701</td>
<td>-0.10</td>
<td>(0.10)</td>
<td>-0.19</td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviations (n=5).

and 5885, Lb. casei L-49, Lb. plantarum 3070 and Lb. delbrueckii subsp. lactis 306701 exhibited prebiotic activities with the EPS besides the parent Lb. plantarum 301102 and mutant strain 301102S.

High growth rates for many LAB strains were shown with GOS (data not shown), but low prebiotic activities were shown with GOS because the growth rate of E. coli was also high. The product used as GOS in the present study contained more than 50% GOS and less than 50% monosaccharide and lactose. Low prebiotic activity scores of GOS observed in the present study may be caused by utilization of monosaccharide and lactose other than the GOS by E. coli.

Low prebiotic activities for tested LAB strains were shown with inulin. This result agrees with earlier reports, and inulin exhibited high prebiotic activity with Bifidobacterium (Huebner, 2007; Gopal et al., 2001).

The highest prebiotic activity score was for strain 301102 with the EPS. This demonstrated that the EPS was utilized specifically by the parent strain 301102. Utilization of prebiotics requires the presence of specific hydrolysis and/or transport systems. Therefore, the parent strain may have enzymes to degrade the EPS from the mutant strain.

Lb. plantarum 301102S can produce EPSs in whey and these EPSs can be used by the probiotic parent strain 301102. Further work is needed to determine the prebiotic action of this EPS in digestive tracts in vivo.

References


rides from Lactobacillus rhamnosus RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-γ in mouse splenocytes. Lait, 81, 683-698.


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