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Characterization of the LP28 strain-specific exopolysaccharide biosynthetic gene cluster found in the whole circular genome of *Pediococcus pentosaceus*

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ABSTRACT

We have previously isolated a lactic acid bacterium (LAB), *Pediococcus pentosaceus* LP28, from the longan fruit *Euphoria longana*. Since the plant-derived LAB strain produces an extracellular polysaccharide (EPS), in this study, we analyzed the chemical structure and the biosynthesizing genes for the EPS.

The EPS, which was purified from the LP28 culture broth, was classified into acidic and neutral EPSs with a molecular mass of about 50 kDa and 40 kDa, respectively. The acidic EPS consisted of glucose, galactose, mannose, and *N*-acetylglucosamine moieties. Interestingly, since pyruvate residue was detected in the hydrolyzed acidic EPS, one of the four sugars may be modified with pyruvate. On the other hand, the neutral EPS consisted of glucose, mannose, and *N*-acetylglucosamine; pyruvate was scarcely detected in the polysaccharide molecule.

As a first step to deduce the probiotic function of the EPS together with the biosynthesis, we determined the whole genome sequence of the LP28 strain, demonstrating that the genome is a circular DNA, which is composed of 1,774,865 bp (1683 ORFs) with a GC content of 37.1%. We also found that the LP28 strain harbors a plasmid carrying 6 ORFs composed of 5366 bp with a GC content of 36.5%. By comparing all of the genome sequences among the LP28 strain and four strains of *P. pentosaceus* reported previously, we found that 53 proteins in the LP28 strain display a similarity of less than 50% with those in the four *P. pentosaceus* strains. Significantly, 4 of the 53 proteins, which may be enzymes necessary for the EPS production on the LP28 strain, were absent in the other four *P. pentosaceus* strains and displayed less than 50% similarity with other LAB species. The EPS-biosynthetic gene cluster detected only in the LP28 genome consisted of 12 ORFs containing a priming enzyme, five glycosyltransferases, and a putative polysaccharide pyruvyltransferase.

1. Introduction

LABs, which are used for various fermented foods, affect human health. The bacteria are major representatives of probiotics, which have been defined by the World Health Organization (WHO) as live microorganisms. In fact, various physiological functions of LABs, such as intestinal-regulation [1–3], blood-pressure-lowering [4,5], anti-bacterial [6–8], anti-tumor [9–11], anti-allergic [12–14], and blood-cholesterol-reducing [15–17] functions, have been reported. These beneficial effects on human health caused by the cell-body material of the LAB itself and/or the second metabolic compound produced by LAB involve the interaction of commensal organisms living in the digestive tract.

Pediococcus pentosaceus has often been isolated from fermented foods and silage. Several strains of *P. pentosaceus* produce anti-bacterial substances [18] and reduce acute liver injury induced by D-galactosamine in rats [19] and encephalitis [20]. We have recently shown that when a plant-derived lactic acid bacterium (LAB), *P. pentosaceus* LP28, was orally administrated to mice with high-fat-diet-induced obesity, the obesity and fatty liver of the mice were improved [21]. These results indicate that the strain is effective against obesity as a risk factor of human metabolic syndrome.

EPSs, produced by several LABs, have recently attracted attention for their physiological functions, such as immune-stimulating abilities [22,23]. We have found that *P. pentosaceus* LP28 produces

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an EPS. In this study, we characterized the LP28-derived EPS and analyzed its chemical components.

On the other hand, whole-genome sequencing is expected to utilize the genetic basis behind the metabolic functioning of LAB for the development of a probiotic application. In this study, we determined the whole genome sequence of *P. pentosaceus* LP28 and compared it with those of four strains (ATCC25745 [24], LI05 [25], SL4 [26], and IE-3 [27]) of *P. pentosaceus*, which have been previously analyzed. The genome sequence information will be useful to understand and utilize the specific probiotic ability of *P. pentosaceus* LP28.

2. Materials and methods

2.1. Strain used

P. pentosaceus LP28 is a lactic acid bacterium that has been isolated from the longan fruit *Euphoria longana* [21]. The strain, which was inoculated into a flask containing de Man, Rogosa and Sharpe (MRS) medium (Becton, Dickinson, and Company), was incubated at 28 °C for 24 h. Glycerol stocks of the strain were prepared by mixing the culture broth with an equivalent 33% glycerol solution and then stored at −80 °C until use.

2.2. Fermentation

The seed culture was done in a flask containing MRS medium at 28 °C for 18 h. The cells, which were harvested by centrifugation at 8000 × *g* for 20 min, were washed twice with the sterile phosphate-buffer saline. The washed cells were inoculated in a semi-defined medium (SDM) [28,29] in a fermenter (Iwashiya Bio-Science, 3 L Mini Jar Fermenter). The yeast nitrogen source in SDM [28] was substituted for a vitamin solution and trace elements solution [29]. The fermentation was carried out with a constant pH 6.5 by automatically adding NaOH solution (25% *w*/*v*) under the condition of 200 rpm agitation and 200 ml/min flow of N_2 gas at 30 °C for 48 h. The content of EPS in the culture broth was analyzed by the phenol-sulfuric acid method.

2.3. Purification of the EPS

Trichloroacetic acid (TCA) was mixed with the LP28 culture broth (final 4% v/v). After being stirred for 30 min at 4 °C, the mixture was centrifuged at 12,500 × *g* for 10 min. An equal volume of acetone was added to the supernatant fluid. After standing overnight at 4 °C, the resulting precipitate, which was collected by centrifugation at 12,500 × *g* for 10 min, was dissolved in 50 mM Tris–HCl (pH 8.0) and centrifuged at 27,000 × *g* for 30 min. A DNase and RNase solution were added to the supernatant fluid (each final concentration is 10 μg/ml). After incubation for 6 h at 37 °C, a proteinase K solution was added (final 20 μg/ml) and followed by incubation for 16 h at 37 °C. TCA was added to the incubation mixture to 10% (v/v) at a final concentration in the ice-cooled condition. After 1 h standing, the supernatant fluid was obtained by centrifugation at 27,000 × *g* for 30 min. After three volumes of 100% ethanol were added to the supernatant fluid, the resulting precipitate was collected by centrifugation at 17,300 × *g* for 5 min and washed with 70% ethanol. The air-dried precipitate was dissolved in purified water and dialyzed by dialysis membrane (MWCO 8000) for 48 h, changing the purified water four times, and recovered by freeze drying. The crude EPS was resuspended in 50 mM Tris–HCl (pH 8.0) and purified by a Toyopearl DEAE-650M column (Toso, 2.5 cm × 22 cm). The neutral EPS was eluted with 50 mM Tris–HCl (pH 8.0), whereas the acidic EPS was obtained by eluting with a NaCl gradient (0 → 0.5 M) in 50 mM Tris–HCl (pH 8.0) at a flow rate of 1.0 ml/min. The eluted samples were analyzed for carbohydrate content by the phenol-sulfuric acid reaction, and the fractions containing EPS were dialyzed against purified water and freeze-dried.

2.4. Calculation of molecular mass of the EPS

The molecular mass of the EPS, which was produced by the LP28 strain, was estimated by gel-filtration chromatography using a Sephacryl S-500 HR (GE Healthcare) equipped in an HPLC system. A solution of 0.1 M $NaNO_3$ was used as a mobile phase at a flow rate of 0.8 ml/min. The elution profile of the EPS was monitored by the RI detector (RI-2031*Plus*, Jasco). The molecular mass was calculated using dextran (Sigma) as an internal standard.

2.5. Analysis of monosaccharide consisting of EPS

Neutral and acidic EPSs (each 5 mg), which were separately dissolved in 1 ml of 2 M trifluoroacetic acid (TFA), were hydrolyzed for 2 h at 120 °C. Each hydrolyzed EPS solution was dried *in vacuo* and dissolved in purified water. Each hydrolysate, which was filtrated with a 0.2 μm pore-sized membrane filter, was applied on an HPLC column chromatography (Thermo Scientific ICS-5000; column: CarboPac PA1, 2 × 250 mm; guard column: CarboPac PA1, 2 × 50 mm; elute solution: 16 mM NaOH; flow rate: 0.25 ml/min; detection: pulsed amperometric electrochemical detector).

2.6. Analysis of pyruvic acid and acetic acid

Neutral and acidic EPSs (2 mg), which were separately dissolved in 1 ml of 2 M TFA, were hydrolyzed for 2 h at 120 °C. The hydrolysates were dried *in vacuo* and dissolved in 3.8 mM H_2SO_4. The existence of pyruvic acid in each hydrolyzed EPS was analyzed by the HPLC method (column: Aminex HPX-87H (Bio-Rad); solvent: 3.8 mM H_2SO_4; detection: UV (210 nm); flow rate: 0.6 ml/min). To confirm the existence of the *N*-acetyl residue bound covalently to the glucosamine molecule, the presence of acetic acid in each hydrolysate of neutral and acidic EPSs was analyzed by using the HPLC method (column: Shodex RS pak, KC-811; solvent: 1.0 mM perchloric acid; detection: Conductivity Detector; flow rate: 1 ml/min).

2.7. Genome DNA extraction

P. pentosaceus LP28 was grown in an MRS medium. The bacterial cells were collected by centrifuging the culture broth. The total DNA from the cells was extracted by using a DNeasy Plant Mini Kit (Qiagen).

2.8. Plasmid DNA extraction

After cultivation in MRS medium, the LP28 cells were collected by centrifugation. Plasmid DNA derived from the cells was extracted by the Genopure Plasmid Maxi Kit (Roche). The bacterial cells, which were suspended in a buffer containing lysozyme (Wako) and achromopeptidase (Wako) (each final concentration is 4 mg/ml), were incubated for 3 h at room temperature to make the cell lysate.

2.9. Genome sequencing and assembly

The paired end library, which was prepared by fragmentizing the genomic DNA, was used for the next-generation sequencing platform Illumina HiSeq 2500 (read length 2 × 75 bp). The mate pair library, which was prepared by fragmentizing the same genomic DNA, was used for the next-generation sequencing platform Roche454FLX Titanium (1/2 run). The read sequence obtained by genomic sequencing was assembled with Newbler v2.8 (the analysis software with Roche454FLX Titanium). The genomic DNA solution