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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

was added on MapCard, digested with a restriction enzyme, and stained with fluorescence. The restriction enzyme map of the whole genome was prepared by measuring the length of each DNA fragment. Contig sequences obtained from the genome assembly were aligned on the restriction enzyme map of the whole genome.

2.10. Genome annotation and comparison

Amino acid sequences of the deduced ORFs were annotated by BLAST searching at the NCBI genome database and compared with the sequences of *P. pentosaceus* ATCC25745 [24], *P. pentosaceus* LI05 [25], *P. pentosaceus* SL4 [26], and *P. pentosaceus* IE-3 [27]. The sequence of low identities that possesses less than 50% similarity with the four *P. pentosaceus* strains was assessed according to similarity with other organisms by BLAST searching.

3. Results

3.1. Purification and characterization of EPS

P. pentosaceus LP28, which was grown in SDM medium (2000 ml) under the control of pH 6.0, produced 234 mg/l of EPS. When the EPS sample was applied on the chromatography using an ion-exchange column (Toyopearl DEAE-650M) and eluted with the NaCl gradient method, two separate peaks were observed, suggesting that the sample contains neutral and acidic EPSs (Fig. 1). The molecular mass of the neutral EPS, which was estimated by gel-filtration column, was approximately 40 kDa, whereas that of the acidic EPS was estimated to be approximately 50 kDa.

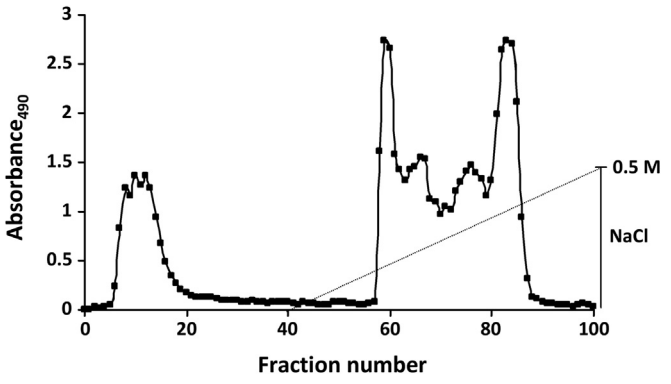

Fig. 1. Fractionation of EPS produced by *Pediococcus pentosaceus* LP28 strain by ion-exchange chromatography. The elute fractions were analyzed for carbohydrate content by the phenol-sulfuric acid reaction.

Analysis of the monosaccharide contained in the hydrolyzed neutral EPS shows the existence of glucose, glucosamine, and mannose. On the other hand, in the hydrolyzed acidic EPS, galactose was detected together with glucose, glucosamine, and mannose (Fig. 2 and Table 1). In addition, acetic acid was confirmed to be present in both hydrolyzed solutions of neutral and acidic EPSs (data not shown).

3.2. Detection of pyruvic acid in the acidic EPS

It was suggested by the genomic analysis that an ORF encoding an enzyme catalyzing the addition of pyruvate residue to EPS is located on a polysaccharide biosynthesis gene cluster in the LP28 strain. By HPLC analysis of the hydrolyzed acidic EPS, in fact, we observed a peak taking the same retention time as that of pyruvic acid, suggesting that the pyruvic acid is a component residue consisting of the acidic EPS (Fig. 3). The organic acid was scarcely detected in the hydrolysate of the neutral EPS.

3.3. Genome sequence analysis

We determined the whole genome sequence analysis of the LP28 strain, demonstrating that the genome is a circular DNA

Table 1
Relative molar compositions of monosaccharide from acidic and neutral EPSs.

Saccharide	RT (min)	Relative molar composition	
		Neutral EPS	Acidic EPS
Glucosamine	11.17	3.5	1.8
Galactose	12.92	–	0.3
Glucose	13.68	4.6	6.6
Mannose	14.72	1.0	1.0

Concentration of each monosaccharide in hydrolyzed EPS solutions were calculated with monosaccharide standard solutions (Thermo Scientific).

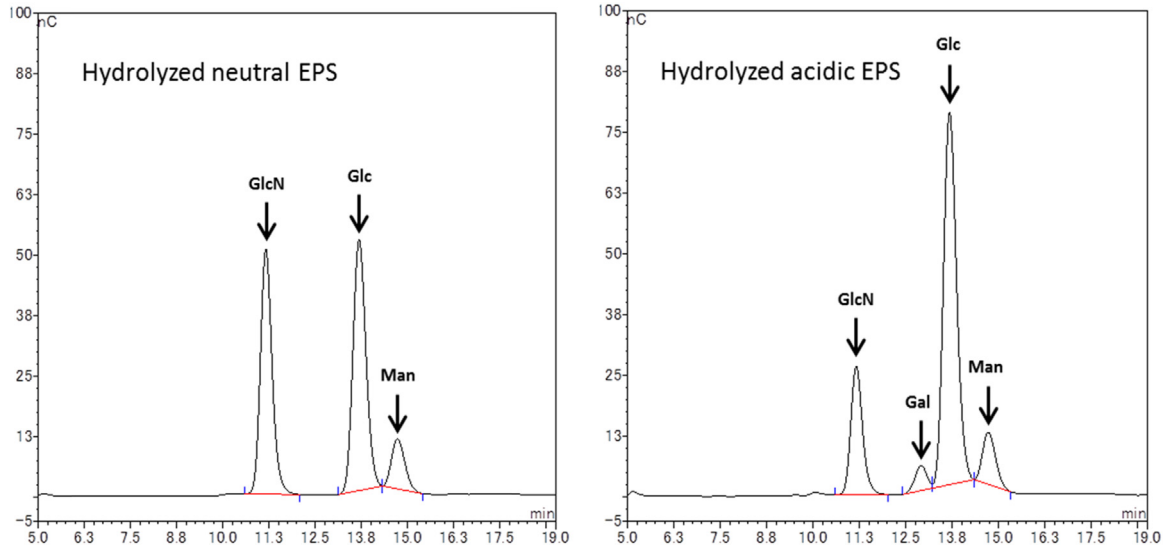

Fig. 2. HPLC chromatogram of monosaccharide from hydrolyzed EPS. The hydrolyzed acidic and neutral EPSs were dissolved in purified water. After the filtration, each hydrolyzed EPS was analyzed by HPLC. Chromatographic peaks were identified by monosaccharide standard solutions (Thermo Scientific). Glc: Glucose, Gal: Galactose, GlcN: Glucosamine, Man: Mannose.

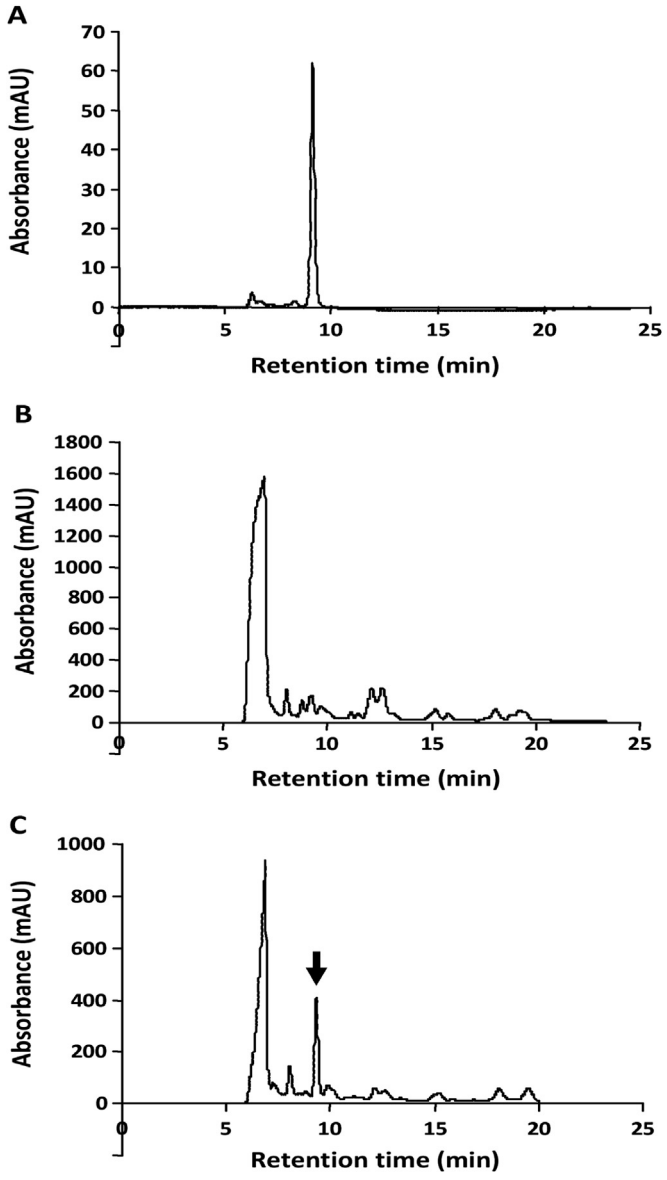

Fig. 3. HPLC analysis of pyruvic acid in the hydrolyzed EPSs from LP28 strain. Hydrolyzed neutral EPS (B), acidic EPS (C) and 1 mM of authentic pyruvic acid (A) were dissolved in 3.8 mM H_2SO_4 and analyzed by the HPLC.

Table 2
Nucleotide content and gene counts of genome and plasmid of *P. pentosaceus* LP28.

	Scaffold 1 (genome)	Scaffold 2 (plasmid)
Number of bases	1,774,865	5366
Number of gaps	25	0
Total length of gaps	22,491	0
Contig GC%	37.1%	36.5%
Number of genes	1740	6
Number of CDS	1683	6
Number of tRNA	51	0
Number of rRNA	6	0

composed of 1,774,865 bp (1683 ORFs) with a GC content of 37.1%. In addition, the strain harbors a plasmid that carries 6 ORFs composed of 5366 bp with a GC content of 36.5% (Table 2). The LP28 whole genome harbors 51 tRNA genes. In addition, the genome contains genes encoding the cholylglycine hydrolase responsible for bile salt resistance, six universal stress proteins (UspA), and the arsenate reductase necessary for some environmental stress resistances. The sequences of ORFs encoding these resistance genes exhibit high similarities to those of four strains of *P. pentosaceus*, such as ATCC25745 [24], LI05 [25], SL4 [26], and IE-3 [27]. The genomic DNA of the LP28 strain also contains the ORFs necessary for the colicin V-producing family protein, which functions as an antibacterial substance.

Comparing proteins deduced from the genome sequence of the LP28 strain and those of the four *P. pentosaceus* strains, 71 ORFs displayed less than 50% similarity. The protein function of 53 of 71 ORFs was assigned through the NCBI database. The function of the remaining 18 proteins cannot be presumed at this time. It should be noted that four ORFs necessary for EPS production, which encode polysaccharide polymerase, sugar phosphotransferase, *N*-acetylglucosaminyl transferase and polysaccharide pyruvyltransferase displayed less than 50% similarity with the four *P. pentosaceus* strains and other LAB species.

Forty-five of 53 protein sequences in the LP28 chromosome, which were assigned for the function, displayed more than 50% similarity with those in several LAB species. The coding sequence for the cellulase catalyzing the digestion of vegetable fiber, which is found on the LP28 genome, exhibits a 71% similarity with that of *Pediococcus lolii* isolated from ryegrass silage [30].

The EPS-biosynthetic genes, which were found in the LP28 genome, are designated *ppeA-L*, and the proteins encoded by them are listed in Table 3. The amino acid sequences of four ORFs (two EPS-biosynthetic proteins, a capsular EPS-biosynthetic protein, and a sugar transferase; PpeA-D) in the LP28 strain displayed high similarity to those deduced in the four *P. pentosaceus* genomes, but another eight ORFs (PpeE-L) did not. One of the eight ORFs was annotated to polysaccharide pyruvyltransferase, which displays a 35% similarity with *Streptococcus constellatus* subsp. *constellatus* SK53 and displays a 31% similarity with *Bifidobacterium longum* 44B [31] and GT15 [32]. A sugar transferase (PpeD) displayed 64% similarity with priming glycosyltransferase of *Lactobacillus plantarum* AY01. Two sugar phosphotransferase (PpeH and PpeI) displayed 51% and 38% similarity with glycosyltransferase of *P. pentosaceus* IE-3, respectively.

In addition, we confirmed by an agarose gel electrophoretic profile of the total DNA that the LP28 strain harbors a plasmid (data not shown). The plasmid size (5.4 kb) corresponds with the DNA length obtained by genome sequence analysis. According to the genome sequence information, the plasmid in the LP28 strain encodes six ORFs. Two of the ORFs are proteins needed for replication and DNA segregation, such as the ATPase FtsK/SpoIIIE–related protein. However, we cannot identify the protein function of the other four ORFs at this time.

4. Discussion

The health functionality of EPSs produced by several LABs is attracting attention. We are interested in whether the obesity and fatty liver of mice induced by a high-fat diet are improved by the oral administration of the EPS from *P. pentosaceus* LP28. It is also significant to evaluate whether the acidic EPS can activate natural killer cells *in vitro*. The property of the EPS can also be known from the existence of some ORFs deduced by the whole genome analysis.

In the present study, the EPSs were dissolved in trifluoroacetic acid, hydrolyzed for 2 h at 120 °C, and then dissolved in 3.8 mM H_2SO_4. By using this method, the acetyl group bound to glucosamine is easily removed. The glucosamine detected in the acidic and neutral EPSs must be *N*-acetylglucosamine. We detected the acetic acid from the hydrolyzed solutions of EPSs. Thus, we conclude that the acidic EPS components are glucose, galactose, mannose, *N*-acetylglucosamine, and pyruvic acid.

Table 3
Sequence comparison of EPS production gene cluster of *P. pentosaceus* LP28.

Protein	Length (aa)	*P. pentosaceus*				BLASTp hit				
		IE-3	SL4	ATCC25745	LI05	Functional description	Organism	Query cover (%)	Identity (%)	Accession number
PpeA	261	98	94	97	97	EPS biosynthesis protein	*Pediococcus pentosaceus* ATCC 25745	100	97	WP 011673151.1
PpeB	243	98	99	99	100	EPS biosynthesis protein	*Pediococcus pentosaceus*	100	100	WP 029257819.1
PpeC	262	98	98	98	97	Capsular EPS biosynthesis protein	*Pediococcus pentosaceus* ATCC 25745	100	98	ABJ 67655.1
PpeD	221	96	99	97	40	Sugar transferase	*Pediococcus pentosaceus* IE3	100	96	WP 002833952.1
PpeE	330	23	62	28	25	Glycosyltransferase family 1	*Pediococcus pentosaceus* SL4	97	62	AHA 04799.1
PpeF	335	27	51	39	27	Glycosyltransferase family 2	*Pediococcus pentosaceus* SL4	96	51	WP 023440188.1
PpeG	385	ND	ND	ND	ND	Polysaccharide polymerase	*Lactobacillus casei* M36	99	35	WP 003588324.1
PpeH	181	51	68	44	48	Sugar phosphotransferase	*Pediococcus pentosaceus* SL4	97	68	WP 023440189.1
PpeI	96	38	49	25	34	Sugar phosphotransferase	*Pediococcus pentosaceus* SL4	94	49	WP 023440189.1
PpeJ	326	27	27	31	27	N-acetylglucosaminyltransferase	*Lactobacillus salivarius* str. Ren	62	35	WP 047034941.1
PpeK	300	ND	ND	ND	ND	Polysaccharide pyruvyltransferase	*Streptococcus constellatus* subsp. *constellatus* SK53	70	35	WP 006270484.1
							Bifidobacterium longum 44B	80	31	EIJ28766.1
							Bifidobacterium longum GT-15	80	31	WP 038426319.1
PpeL	458	ND	ND	23	23	Exopolysacharide protein Wzx	*Lactobacillus pentosus* IG1	100	63	CCC 16059.1
						Flippase Wzx	*Lactobacillus buchneri* CD034	95	42	WP 014940887.1

ND: Not detected the sequence which possess more than 30% query cover and more than 50% similarity simultaneously.

The pyruvic acid bound to EPS can be found in a gram-negative bacterial EPS [33]. It is known as a configuration of pyruvic acid linked to glycosyl residue as a cyclic acetal. Production of the pyruvic acid-bound EPS has been reported in *Lactobacillus rhamnosus* RW-9595M [34] and *B. longum* JBL05 [35] but not in *P. pentosaceus*. The pyruvic acid-bound EPS possesses cytokine-activating ability [36]. It is significant to know whether the acidic EPS produced by the LP28 strain possesses cytokine-activating ability.

It has been widely recognized that both of probiotics and gut microbiota play a role on human gut homeostasis. In addition, extracellular molecules such as bacteriocin, lactic acid, and EPS, which are produced by the probiotic bacteria, also exhibit beneficial effects to the host. It is important to know that whether the anti-obesity effect of *P. pentosaceus* LP28 is brought by EPS which is produced by the LAB strain. The confirmation by an experiment using the obese mice is in progress.

The complete genome sequence and plasmid sequence of *P. pentosaceus* LP28 has been deposited in GenBank under accession number DF970691 and LC075345, respectively.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.01.004.

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