Regular Article

Characterization of the SN35N Strain-Specific Exopolysaccharide Encoded in the Whole Circular Genome of a Plant-Derived *Lactobacillus plantarum*

Masafumi Noda,[a] Masaya Shiraga,[a] Takanori Kumagai,[b] Narandalai Danshiitsoodol,[a] and Masanori Sugiyama*[,a]

[a] *Department of Probiotic Science for Preventive Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan: and* [b] *Department of Microbiology, Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan.*

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Lactobacillus plantarum **SN35N, which has been previously isolated from pear, secretes exopolysaccharide (EPS). The aim of the present study is to characterize the EPS chemically and to find the EPS-biosynthesizing gene cluster. The present study demonstrates that the strain produces an acidic EPS carrying phosphate residue, which is composed of glucose, galactose, and mannose at a molecular ratio of 15.0 : 5.7 : 1.0. We also show that acidic EPS strongly inhibits the catalytic activity of hyaluronidase (EC 3.2.1.35), promoting an inflammatory reaction. In the present study, we also determined the complete genome sequence of the SN35N strain, demonstrating that the genome is a circular DNA with 3267626 bp, and the number of predicted coding genes is 3146, with a GC content of 44.51%. In addition, the strain harbors four plasmids, designated pSN35N-1, -2, -3, and -4. Although four EPS-biosynthesizing genes, designated *lpe1*, *lpe2*, *lpe3*, and *lpe4*, are present in the SN35N chromosomal DNA, another EPS gene cluster, *lpe5*, is located in the pSN35N-3 plasmid, composed of 35425 bp. EPS low-producing mutants, which were obtained by treating SN35N cells with novobiocin, lost the *lpe5* gene cluster in the plasmid-curing experiment, suggesting that the gene cluster for the biosynthesis of acidic EPS is present in the plasmid. The present study shows the chemical characterization of the acidic EPS and its inhibitory effect to the hyaluronidase.**

Key words anti-hyaluronidase activity; exopolysaccharide; *Lactobacillus plantarum*; whole-genome sequence

Probiotics are defined as "living microorganisms conferring a health benefit to the host, when administered in adequate amounts."[1] Lactic acid bacterium (LAB), some strains of which are known as a probiotic, is a generic name given to non-pathogenic Gram-positive bacteria that produce one or two moles of lactic acid from one mole of sugars during fermentation. LABs, which are also generally recognized as safe (GRAS) microorganisms, have been traditionally used to make fermented foods such as fermented dishes, yogurt, and cheese.[2–4]

Some LAB strains give health benefits to human, such as immunomodulation and improvement of intestinal disorders. It has been also reported that an LAB strain has a potent effect on preventing and improving obesity, and helps to decrease serum lipids and cholesterol.[5–8] Until now, we have established a plant-derived LAB library which consist of more than 600 strains from medicinal plants, vegetables, flowers, and fruits.[9–11] We have searched LAB strains useful for preventive medicine and found several strains that enhance intestinal immunity, improve liver function, and prevent metabolic syndrome.[12–15]

Exopolysaccharide (EPS) produced by some LAB strains[16,17] exhibits immunomodulation, anti-gastritis and anti-ulcer functions, and anti-virus activities.[18–20] Although LAB strains numbered SN13T and SN35N[13] isolated previously by our group were identified as *Lactobacillus* (*Lb.*) *plantarum*, only the SN35N strain produces EPS. We have also isolated two EPS-producing *Pediococcus* (*P.*) *pentosaceus*, named LP28 and LY45 strains. We have found using high-fat diet-induced obese mice that the intake of the LP28 strain effective for reducing body weight gain, improving fatty liver, and decreasing accumulated abdominal visceral fat.[15] In addition, we have previously determined the strain-specific EPS biosynthetic gene cluster in EPS-producing *P. pentosaceus* LP28.[21] On the other hand, the EPS produced by LY45 strain has been found to inhibit the enzymatic activity of hyaluronidase.[22] Hyaluronic acid, which is generated by digestion with hyaluronidase, stimulates the inflammatory response reaction.[23,24] Therefore, a compound (substance) that inhibits hyaluronidase may become a candidate for an anti-inflammatory agent.

In the present study, we have analyzed the monosaccharide components and the acidic residue of the acidic EPS produced by a pear-derived *Lb. plantarum* SN35N. Additionally, we evaluate partially the healthcare function, demonstrating that the SN35N-derived acidic EPS strongly inhibits the catalytic activity of hyaluronidase promoting inflammatory reactions. Furthermore, we have found EPS-biosynthesizing gene clusters on the SN35N chromosome and on a plasmid by sequencing the whole genome together with the plasmid DNA. To generate a mutant that scarcely produces EPS, we treated the SN35N cells with novobiocin, demonstrating that the pSN35N-3-cured mutant did not produce the acidic EPS. A gene cluster necessary for the biosynthesis of the acidic EPS

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* To whom correspondence should be addressed. e-mail: sugi@hiroshima-u.ac.jp

may be present on the plasmid.

MATERIALS AND METHODS

Media and Culture Conditions De Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) was used as a culture medium for *Lb. plantarum* SN35N. A semi-defined medium (SDM)[25] supplemented with a 0.2% (v/v) vitamin solution and a 0.1% (v/v) trace element solution[26] instead of a yeast nitrogen base, called modified SDM, was used to produce the EPS.

Culture Condition for Producing EPS For the seed culture, a portion of the SN35N cells frozen stock solution was inoculated into fresh MRS broth and grown at 37°C until the stationary phase of growth under the condition of standing culture. The seed culture was inoculated at 0.5% (v/v) into a modified SDM medium and incubated at 28°C for 2 d under the stand culture condition without shaking.

Purification of EPS from the SN35N Culture Broth EPS was purified from the culture broth in accordance with the method described previously[22]: After the addition of trichloroacetic acid (TCA) to the SN35N culture broth, the LAB cell mass and proteins were removed from the cultured broth by centrifugation. The resulting supernatant fluid was mixed with acetone to precipitate the EPS. The nucleotides and proteins in the precipitated EPS were digested with deoxyribonuclease I (Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.), ribonuclease A (Nacalai Tesque, Kyoto, Japan), and proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After adding the TCA, the protein and debris were removed by centrifugation, and the crude EPS was obtained from the resulting supernatant fluid by ethanol precipitation. The resulting EPS pellet was dissolved into distilled water. Prior to determination of the EPS content by the phenol sulfate method,[27] the crude EPS solution was dialyzed against the distilled water using an Amicon Ultra (MWCO=10 kDa, Merck Millipore Ltd., Carrigtwohill, Co., Cork, Ireland).

The acidic EPS was purified from the crude EPS by using a TOYOPEARL DEAE-650M column (2.5×22 cm; Tosoh Bioscience, Tokyo, Japan) with the method described previously.[22] The acidic EPS was eluted from the column with a linear gradient of NaCl (0 to 240 min, 0 mM; 240 to 600 min, 0–500 mM). The EPS-contained fractions were pooled and dialyzed against the distilled water by using an Amicon Ultra (MWCO=10 kDa).

Hyaluronidase Inhibitory Assay The assay for hyaluronidase inhibition was performed according to the protocol established by Fujitani *et al.*,[28] with a slight modification method.[22] The inhibitory activity was compared by calculating the IC_{50} value, which is defined as the EPS concentration inhibiting 50% enzyme activity.

Acute Toxicity Using Rats and Mutagenicity Tests An acute oral toxicity experiment of the SN35N cells, which was orally administrated, was done through the New Drug Development Research Center, Inc. (the protocol numbers are 06060-1 and 06060-2). The experiment was performed according to the Principles of Good Laboratory Practice. Five-week-old Crl: Ceasarean Derived (Sprague–Dawley) male rats were purchased from Charles River Laboratories Japan, Inc. LAB cells were resuspended into the purified water (Yakuhan Pharmaceutical Co., Ltd., Hokkaido, Japan). The rats were divided into three groups of five rats each and housed in stainless steel cages under controlled temperature (22±3°C) and 12 h light-dark cycles. Rats had free access to CRF-1 diets (Oriental Yeast Co., Ltd., Tokyo, Japan) and water. After 1 week of acclimation, each group was assigned an LAB-fed group and a reference group: a high-dose group (1.5×10^{12} colony-forming unit (CFU)/kg), a low-dose group (0.75×10^{12} CFU/kg), and a pure water group. Cell suspensions or purified water was orally administrated to rats by using a sterile probe once a day for 2 weeks. During the experiment, rats' exercise activity, behavior, general health status, and body weight were recorded at 1-, 3-, 7-, 10-, and 14-d points. After the experimental period, the rats were euthanized, and histological analyses of some extracted organs were performed. The same experiment was also carried out using female rats.

The mutagenicity test (*umu* test) of the *Lb. plantarum* SN35N culture broth was performed using an Umulac AT-*F* kit (Protein Purify Co., Ltd., Gunma, Japan) in accordance with the manufacturer's instructions.

Molecular Mass Analysis The molecular mass of the acidic EPS was estimated using an HPLC system equipped with gel-filtration chromatography with a SUGAR KS-806 column (Showa Denko, Tokyo, Japan). The analytical conditions were as follows: ultrapure water was used as a mobile phase at a flow rate of 0.7 mL/min. The column oven temperature was set at 80°C, and the eluent was monitored by the RI detector. The molecular mass was calculated from the calibration curve made using pullulan standards.

Monosaccharide Composition of EPS The composition of monosaccharide in the acidic EPS was analyzed after the hydrolysis reaction as follows: the purified EPS was dissolved in 10 mL of purified water. After adding 300 μL of 18 M H_2SO_4, the samples were hydrolyzed for 3.5 h at 110°C. The hydrolysate was neutralized by adding $Ba(OH_2)\cdot 8H_2O$; it was then filtrated with a 0.45 μm pore-sized membrane filter. The resulting filtrate was applied on an HPLC system equipped with a SUGAR SP0810 column (Showa Denko). The analytical conditions were as follows: ultrapure water was used as a mobile phase at a flow rate of 0.7 mL/min. The analysis was performed at 80°C, and the eluates from the column were monitored with an RI detector.

Detection of Phosphate Residue BIOMOL Green Reagent (Biomol GmbH, Hamburg, Germany) was used to analyze the phosphate residue in acidic EPS. The EPS was dissolved into distilled water at a final concentration of 0.02% (w/v). Five milliliter of EPS solution mixed with 1 mL of 40 g/L potassium peroxodisulfate was incubated at 120°C for 30 min, and then cooled to room temperature (r.t.). A sample not subjected to heat treatment was used as a control. In this case, distilled water was used instead of the EPS solution as a blank test sample.

One milliliter of BIOMOL Green Reagent and a 100 μL aliquot of 4-times diluted measuring samples were mixed and reacted at r.t. for 25 min. The phosphate release was confirmed by measuring A_{620}. The quantity of phosphate was calculated form the standard curve prepared by using serial dilutions of phosphate with 5–80 μM.

DNA Preparation The SN35N chromosomal DNA was isolated as described previously,[10] with a slight modification: the cell mass was harvested from the culture broth by

centrifugation and washed with a glucose–ethylenediaminetetraacetic acid (EDTA) buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris–HCl, pH 8.0). The washed cells were resuspended into the same buffer, containing 10 μg/mL ribonuclease A (Nacalai Tesque), 4 mg/mL lysozyme (Wako Pure Chemical Industries, Ltd.), and 0.4 mg/mL achromopeptidase (Wako Pure Chemical Industries, Ltd.). After incubation for 3 h at 37°C, the cells were lysed by adding 0.1 volume of 10% (w/v) sodium dodecyl sulfate, and the proteins were denatured and removed by chloroform/isoamyl alcohol extraction. Finally, the chromosomal DNA was purified by ethanol precipitation.

Genome Sequencing and Annotation The whole genome of the SN35N strain was sequenced on a next-generation sequencing platform, PacBio RS II (Pacific Biosciences, Menlo Park, CA, U.S.A.), on a single molecule real-time (SMRT) cell using P6 polymerase and C4 chemistry (P6C4); the purified genomic DNA was fragmented using a g-TUBE (Covaris, Woburn, MA, U.S.A.), and the sheared short fragments were then purified using an AMPure PB kit (Pacific Biosciences). A PacBio DNA Template Prep Kit 1.0 (Pacific Biosciences) and a PacBio DNA/Polymerase Binding Kit P6 (Pacific Biosciences) were used for constructing the DNA library. The inadequate short fragments were eliminated by Blue Pippin (Sage Science, Beverly, MA, U.S.A.), and the resulting purified DNA library was then sequenced on the PacBio SMRT platform. The hierarchical genome assembly process (HGAP) protocol[29] was used for *de novo* assembling, and the resulting genome contig was annotated by the Microbial Genome Annotation Pipeline (MiGAP). The genome sequence was analyzed using *in silico* Molecular Cloning Genomics Edition (*In Silico* Biology, Inc., Kanagawa, Japan). The GenBank/EMBL/DDBJ accession numbers for the sequences reported in the present study are AP018405 (for chromosomal DNA), AP018406, AP018407, AP018408, and AP018409 (for plasmid DNAs).

Generation of an EPS Non-producing Mutant A mutant from *Lb. plantarum* SN35N, which cannot produce EPS, was isolated by treatment with novobiocin; an aliquot of the overnight culture was inoculated into MRS broth supplemented with 0.8 μg/mL novobiocin, useful as a curing agent for plasmid. After cultivation for 2 d, a portion of the culture was plated on fresh MRS agar and incubated anaerobically for 2 d until colonies appeared. The existence of an *lpe5* cluster in each colony was confirmed by PCR analysis, using sense (5′-catcgcaattatgatcaagcgcg-3′) and anti-sense (5′-gacgctgct-

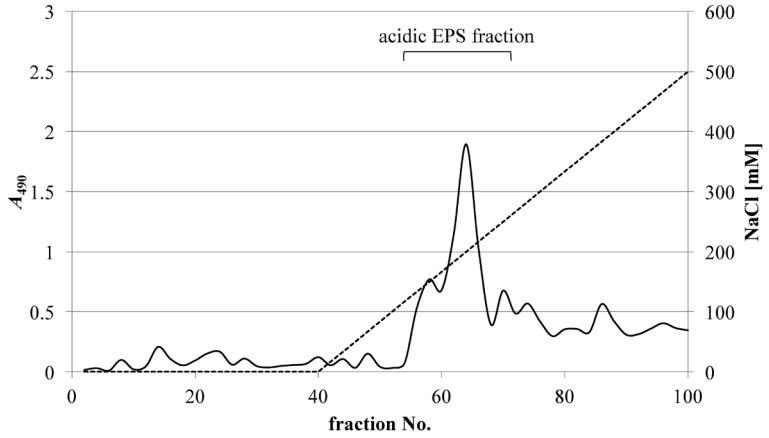

Fig. 1. Anion-Exchange Chromatographic Profiles of the EPS Purified from *Lb. Plantarum* SN35N Using a TOYOPEARL DEAE-650M Column

Dashed lines indicate the NaCl gradient concentrations in the eluates. The presence of EPS was monitored at 490 nm by the phenol sulfate method (solid line).

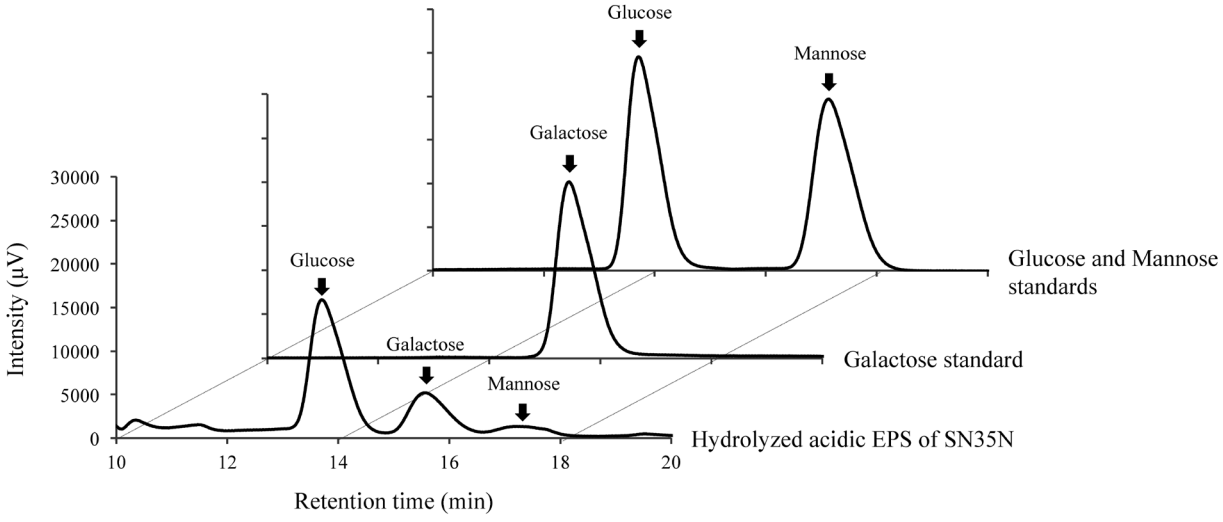

Fig. 2. HPLC Chromatographic Profile of Each Monosaccharide Contained in the Hydrolyzed Acidic EPS of SN35N

Each monosaccharide peak was identified using the monosaccharide standard solution.

tagcatactcacta-3′) primers. The EPS productivity by the plasmid-cured (EPS non-producing) mutant, which was named (SN35N-Δp3), was also evaluated by the same method as for SN35N parental strain.

RESULTS

Characteristics of SN35N EPS Secreted in Culture Broth *Lb. plantarum* SN35N grown in a modified-SDM medium produces EPS outside the cells. The chromatography profile when using a TOYOPEARL DEAE-650M anion-exchange column shows that the SN35N-derived EPS displays an acidic property. The yield of acidic EPS from the culture broth was estimated to be 48.2 mg/L (Fig. 1). The molecular mass, as estimated by a gel-filtration HPLC profile, was approximately 250 kDa.

As shown in Fig. 2 and Table 1, the HPLC profile shows that the acidic EPS is composed of glucose, galactose, and mannose at a monosaccharide ratio of 15.0 : 5.7 : 1.0, respectively. Thus, mannose is slightly detectable in the SN35N-derived EPS. Monosaccharides mainly present in EPS produced by other LAB strains are glucose and galactose.[32] Using phosphate quantification analysis of acid-hydrolyzed acidic EPS, we show that the acidic EPS from the SN35N strain contains 1.0 μmol phosphorylate residue per 1 mg of EPS.

Anti-hyaluronidase Activity of SN35N-Derived EPS We carried out a kinetic analysis to evaluate hyaluronidase's inhibitory effect on the SN35N-derived acidic EPS. The IC_{50} value (240 μg/mL) was lower than that of fucoidan (from *Laminaria Japonica*, 2000$<\mu$g/mL). On the other hand, that of the *P. pentosaceus* LY45-derived acidic EPS, which was previously isolated by our group, was 1300 μg/mL.[22] The IC_{50} values of sodium cromoglicate and dipotassium glycyrrhizinate, which are well-known as anti-inflammatory agents, were 100 and 530 μg/mL, respectively.[22]

Safety Evaluation of SN35N Strain Acute oral toxicity tests for the SN35N cells demonstrated that significant activity changes and feeding-related illness or death of rats were not observed. Obvious differences and inflammatory symptoms were also not observed in some organs in the rats. In addition, a mutagenicity test for the SN35N culture broth did not give rise to mutagenicity.

Table 1. The EPS Component Monosaccharides and Relative Molar Ratio

Saccharide	r.t. (min)	Relative molar ratio
Glucose	13.70	15.0
Galactose	15.55	5.7
Mannose	17.21	1.0

Genome Sequence of the SN35N Strain We determined the whole-genome sequence of the SN35N strain. The sequence information indicates that the whole genome is a circular DNA, and the size is 3267626 bp, with a GC content of 44.51%. The number of CDS (coding sequence) is predicted to be 3146 (Table 2), when compared with other eight *Lb. plantarum* strains deposited in the DDBJ database, in addition to the data published previously.[30,31,33–37] The average genome size of several *Lb. plantarum* strains is 3286 kb, with 44.36% of GC content and 3057 CDS, showing that the values of other *Lb. plantarum* genomes are almost the same as that of the SN35N genome.

EPS-Biosynthesizing Gene Cluster Detected in the *Lb. plantarum* SN35N Genome and Its Own Plasmid The predicted EPS-biosynthesizing gene clusters, designated *lpe1*, *pe2*, *lpe3*, and *lpe4*, are present in the chromosomal DNA (Table 3 and Fig. 3). *Lpe1*, which is smallest of the four clusters, contains only four open reading frames (ORFs) predicted to be involved in EPS biosynthesis (*lpe1A*, *lpe1B*, *lpe1C*, and *lpe1D*). The *lpe2* and *lpe3* clusters, which are found on a region adjacent to *lpe1*, are composed of 10 (*lpe2A–J*) and 11 (*lpe3A–K*) ORFs, respectively. The *lpe4* cluster is 880 kb away from the *lpe1–3* region. Among these clusters, only the *lpe2* and *lpe4* seem to include genes necessary for the biosynthesis of EPS—the priming glycosyltransferase (*lpe2E* and *lpe4E*), glycosyltransferase (*lpe2F*, *lpe2H*, *lpe2I*, *lpe4F*, *lpe4G*, and *lpe4I*), flippase (*lpe2A*, *lpe2J*, and *lpe4J*), polymerase (*lpe2G* and *lpe4H*), and chain length regulators (*lpe2B*, *lpe2C*, *lpe2D*, *lpe4A*, *lpe4B*, and *lpe4C*).

In addition to these four clusters found on the chromosomal DNA, the *lpe5* gene cluster, with a 22 kb size, is present on the plasmid pSN35N-3. As shown in Table 3 and Fig. 4A, the *lpe5* cluster is composed of 16 ORFs (*lpe5A–P*) and contains 11 genes deduced to encode transposase around the cluster. Judging from the annotation information, the ORFs contained in the *lpe5* cluster may be necessary for EPS biosynthesis, as well as the *lpe2* and *lpe4* clusters. However, we could not find a gene encoding phosphotransferase necessary for the phosphorylation of EPS in the cluster.

Plasmid-Curing Experiment To confirm whether genes for the biosynthesis of SN35N-derived EPS are present on the plasmid, *Lb. plantarum* SN35N cells were incubated with novobiocin used as a plasmid-curing agent. PCR analysis was carried out for a mutant that cannot produce the EPS. The result suggests that a DNA fragment containing *lpe5J*, which encodes a putative priming glycosyltransferase, in the EPS

Table 2. Nucleotide and Genomic Features of *Lb. plantarum* Strains

Strain	Genome size (bp)	GC content (%)	CDS	tRNA genes	rRNA genes	Isolation origin	Accession no.	Reference
SN35N	3267626	44.5	3146	75	16	Pear	AP018405	This study
WCFS1	3308273	44.5	3013	70	15	Human saliva	NC_004567	34
JDM1	3197759	44.7	2904	61	16	Human intestinal tract	NC_012984	33
ZJ316	3203964	44.4	2894	61	15	Healthy newborn fecal sample	NC_020229	30
16	3044678	44.7	2784	66	16	Malt production steep water	NC_021514	31
B21	3284260	44.5	3021	63	17	Vietnamese fermented sausage (nemchua)	NZ_CP010528	36
HFC8	3067675	44.3	2766	68	16	Faecal sample	NZ_CP012650	35
KP	3418468	44.3	3184	81	16	Whole fly	NZ_CP013749	37
DF	3423963	44.4	3204	81	16	Whole fly	NZ_CP013753	37

Table 3. Gene Organizations of EPS Biosynthesis Gene Clusters (*lpe1–5*) of *Lb. plantarum* SN35N

Cluster and gene	Size (aa)	Location	Predicted function	Best BLAST match	Source organism	Accession No.	Identity (%)
lpe1 cluster							
lpe1A	323	593342–594313 C	Glycosyltransferase	Hypothetical protein	*Lb. plantarum*	WP_053338792	100
lpe1B	390	592170–593342 C	CDP-glycerol–glycerophosphate glycerophosphotransferase	Hypothetical protein	*Lb. plantarum*	WP_027821174	100
lpe1C	392	590311–591489 C	Glycosyltransferase	Glycosyltransferase family 1 protein	*Lb. plantarum*	WP_044430140	100
lpe1D	440	588992–590314 C	Glycosyltransferase	Hypothetical protein	*Lb. plantarum*	WP_053338793	100
lpe2 cluster							
lpe2A	507	580323–581846	Flippase Wzx	Transporter	*Lb. plantarum*	OBS43084	99.8
lpe2B	255	578507–579274 C	Chain-length determinant Wzz	Polysaccharide biosynthesis protein	*Lb. plantarum*	KWT43483	100
lpe2C	242	577767–578495 C	Tyrosine-protein kinase Wze	Exopolysaccharide biosynthesis protein	*Lb. plantarum*	WP_024971390	100
lpe2D	278	577007–577843 C	Protein-tyrosine phosphatase Wzb	Protein-tyrosine-phosphatase	*Lb. plantarum*	ARW35023	95.5
lpe2E	218	576301–576957 C	Priming glycosyltransferase	Sugar transferase	*Lb. plantarum*	WP_044430148	100
lpe2F	289	575219–576088 C	Glycosyltransferase	Hypothetical protein	*Lb. plantarum*	WP_044430150	100
lpe2G	418	573947–575203 C	Polymerase Wzy	Hypothetical protein	*Lb. plantarum*	WP_044430151	99.5
lpe2H	269	573134–573943 C	Glycosyltransferase	Hypothetical protein	*Lb. plantarum*	WP_053338795	99.6
lpe2I	237	572424–573137 C	Glycosyltransferase	Glycosyltransferase family 2	*Lb. plantarum*	WP_044430157	100
lpe2J	473	569872–571293 C	Flippase Wzx	Hypothetical protein	*Lb. plantarum*	WP_080333751	100
lpe3 cluster							
lpe3A	302	565206–566114 C	Glycosyltransferase	Glycosyl transferase family 2	*Lb. plantarum*	WP_053338797	100
lpe3B	310	564240–565172 C	Glycosyltransferase	Glycosyl transferase family 2	*Lb. plantarum*	WP_053338798	100
lpe3C	377	562594–563727 C	UDP-galactopyranose mutase	UDP-galactopyranose mutase	*Lb. plantarum*	WP_003644178	100
lpe3D	362	561445–562533 C	Tyrosine-protein kinase transmembrane module Wzd	Hypothetical protein	*Lb. plantarum*	WP_053338799	100
lpe3E	207	560816–561439 C	Tyrosine-protein kinase Wze	Hypothetical protein HMPREF0531_11724	*Lb. plantarum*	EFK29287	100
lpe3F	406	559609–560829 C	Polymerase Wzy	Hypothetical protein	*Lb. plantarum*	WP_003644181	100
lpe3G	369	558503–559612 C	Unknown	Hypothetical protein	*Lb. plantarum*	WP_003644182	100
lpe3H	359	557440–558519 C	*O*-Acetyltransferase	Acetyltransferase	*Lb. plantarum*	WP_003644183	100
lpe3I	258	556528–557304 C	Glycosyltransferase	Exopolysaccharide biosynthesis protein	*Lb. plantarum*	WP_021356757	100
lpe3J	472	554920–556338	Flippase Wzx	Flipplase	*Lb. plantarum*	WP_053338801	100
lpe3K	225	553806–554483	Priming glycosyltransferase	Sugar transferase	*Lb. plantarum*	WP_075060689	99.6
lpe4 cluster							
lpe4A	252	2975111–2975869	Tyrosine-protein kinase transmembrane module Wzd	Polysaccharide biosynthesis protein	*Lb. plantarum*	WP_027821336	100
lpe4B	235	2975887–2976594	Tyrosine-protein kinase Wze	Exopolysaccharide biosynthesis protein	*Lb. plantarum*	WP_003640787	100
lpe4C	273	2976533–2977354	Protein-tyrosine phosphatase Wzb	Protein-tyrosine phosphatase	*Lb. plantarum*	CDN27632	100
lpe4D	313	2977370–2978311	UDP-glucose 4-epimerase	Epimerase	*Lb. plantarum*	WP_053338960	100
lpe4E	221	2978298–2978963	Priming glycosyltransferase	Capsular polysaccharide biosynthesis protein	*Lb. plantarum*	AOG30978	100
lpe4F	363	2978963–2980054	Glycosyltransferase	Glycosyl transferase family 1	*Lb. plantarum*	WP_053338958	99.7
lpe4G	342	2980070–2981098	Glycosyltransferase	Glycosyl transferase family 1	*Lb. plantarum*	WP_027822102	100
lpe4H	424	2981095–2982369	Polymerase Wzy	Hypothetical protein	*Lb. plantarum*	WP_027822103	100
lpe4I	322	2982354–2983322	Glycosyltransferase	Glycosyl transferase family 2	*Lb. plantarum*	WP_027822104	100
lpe4J	324	2983879–2984853	Flippase Wzx	Flipplase	*Lb. plantarum*	WP_027822105	100
lpe5 cluster (on the plasmid pSN35N-3)							
lpe5A	374	979–2103 C	Glycosyltransferase	Glycosyltransferase family 2 protein	*Bacillus coagulans*	WP_051357575	42.9
lpe5B	485	7363–8820 C	Flippase Wzx	Flipplase	*Lb. plantarum*	WP_063487733	99.0
lpe5C	169	9977–10486 C	Glycosyltransferase	Glycosyl transferase	*Bacillus cereus*	WP_033687572	41.9
lpe5D	263	10483–11274 C	Glycosyltransferase	Hypothetical protein	*Lb. plantarum*	WP_080283862	46.1
lpe5E	302	11312–12220 C	Polymerase Wzy	EpsG family protein	*Lb. vaginalis*	WP_003717951	30.5
lpe5F	338	12446–13462 C	Glycosyltransferase	Glycosyltransferase family 2 protein	*Clostridium clariflavum*	WP_014255924	43.3
lpe5G	256	13452–14222 C	CDP-alcohol phosphatidyltransferas	Hypothetical protein	*Lb. sakei*	WP_082267650	57.2
lpe5H	149	14209–14658 C	Glycerol-3-phosphate cytidylyltransferase	Glycerol-3-phosphate cytidylyltransferase	*Leuconostoc carnosum*	WP_014974067	75.7
lpe5I	382	14661–15809 C	Glycosyltransferase	Hypothetical protein	*Leuconostoc mesenteroides*	WP_071952261	44.7
lpe5J	231	15836–16531 C	Priming glycosyltransferase	Sugar transferase	*Lactobacillus coryniformis*	WP_010014297	88.7
lpe5K	312	16578–17516 C	UDP-glucose 4-epimerase	UDP-glucose 4-epimerase	*Lb. plantarum*	WP_020923878	86.2
lpe5L	96	17541–17831 C	Protein-tyrosine phosphatase Wzb (truncated)	Tyrosine protein phosphatase	*Lactobacillus* sp.	WP_010495923	96.2
lpe5M	262	19596–20384 C	Protein-tyrosine phosphatase Wzb	Polysaccharide biosynthesis protein, phosphotyrosine-protein phosphatase	*Lb. plantarum*	AGS26747	98.5
lpe5N	242	20356–21084 C	Tyrosine-protein kinase Wze	Exopolysaccharide biosynthesis protein	*Lb. plantarum*	WP_072539917	99.2
lpe5O	255	21096–21863 C	Tyrosine-protein kinase transmembrane module Wzd	Chain length regulator	*Lb. paraplantarum*	CDF77689	98.0
lpe5P	154	22111–22575 C	Glycosyltransferase (truncated)	Hypothetical protein	*Lb. collinoides*	WP_063285095	100

non-producing mutant SN35N-Δp3 was not detected. In addition, the EPS productivity of the mutant was drastically decreased (the EPS productivity of wild-type strain: 48.2 mg/L) until 3.3 mg/L. There results suggest that the *lpe5* gene cluster present on the pSN35N-3 plasmid is involved in the production of the EPS.

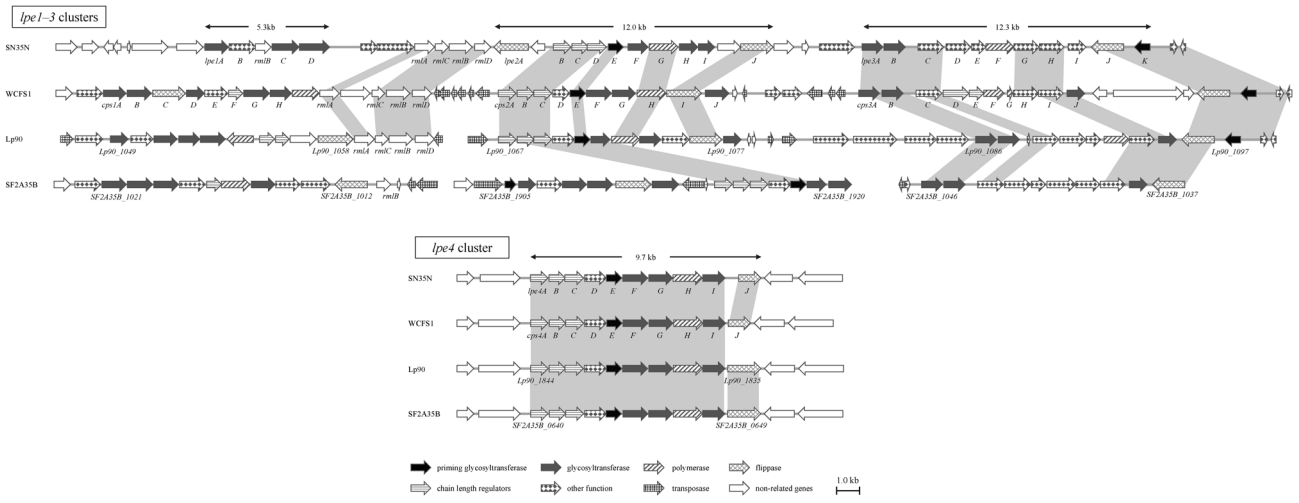

Fig. 3. Genetic Organizations of the EPS Biosynthesis Gene Clusters on the *Lb. plantarum* SN35N (Accession No. AP018405), *Lb. plantarum* WCFS1 (NC_004567), *Lb. plantarum* Lp90 (NZ_JIBX01000000), and *Lb. plantarum* SF2A35B (NZ_LMVD01000000) Genomes

The gene organizations and comparisons of the gene clusters among four *Lb. plantarum* strains are indicated. The gray connecting areas indicate highly conserved regions among the indicated strains.

Table 4. The Sugar Compositions of EPSs Produced by Several *Lb. plantarum* Strains

Strain	Composition sugars and ratio							Reference
	Glucose	Galactose	Mannose	Fructose	Rhamnose	Glucosamine	Galactosamine	
SN35N	14.3	5.7	1.0	—	—	—	—	This study
70810	—	1.0	—	—	—	—	—	38
KF5	4.99	6.9	1.0	—	—	—	—	39
YW32	4.2	4.1	8.2	1.0	—	—	—	40
BC-25	6.0	1.79	92.21	—	—	—	—	41
YW11	2.7	1.0	—	—	—	—	—	42
WCFS1	28	17	—	—	5.0	3.0	—	43
SF2A35B	3.0	66.6	—	—	—	—	313	43
Lp90	3.9	22.2	—	—	25	24.5	24.4	43

DISCUSSION

Table 4 shows the sugar compositions of some *Lb. plantarum* strain-derived EPSs. EPS produced by *Lb. plantarum* 70810 is composed of only galactose as a monosaccharide.[38] However, in the case of *Lb. plantarum* KF5, the produced EPS is composed of mannose, glucose, and galactose at a ratio of 1 : 4.99 : 6.9.[39] Wang *et al.* have reported that the sugar components of EPS produced by *Lb. plantarum* YW32 were mannose, fructose, galactose, and glucose at a ratio of 8.2 : 1 : 4.1 : 4.2.[40] According to another research group, the EPS produced by *Lb. plantarum* BC-25 was composed of glucose, galactose, and mannose at a molar ratio of 6.0 : 1.79 : 92.21.[41] On the other hand, *Lb. plantarum* YW11, which has been isolated from Tibetan Kefir, produces EPS composed of glucose and galactose at a molar ratio of 2.7 : 1, with the possible presence of *N*-acetylated sugar residues in the polysaccharide.[42] However, until now, the EPS-biosynthesizing gene cluster in both strains has not been analyzed.

On the other hand, the EPS-biosynthesizing gene clusters for the WCFS1, SF2A35B, and Lp90 strains, which belong to *Lb. plantarum*, have been analyzed together with the EPS components.[43] The EPS from the WCFS1 strain contains glucose, galactose, glucosamine, and rhamnose at a ratio of 28 : 17 : 3 : 5. The SF2A35B strain produces an EPS composed of galactose and galactosamine, but little glucose (66.6 : 313 : 3, respectively). The monosaccharide component ratio of EPS produced by *Lb. plantarum* Lp90 is composed of glucosamine, galactose, galactosamine, and rhamnose at almost the same, but with a small ratio of glucose (24.5 : 22.2 : 24.4 : 25.0 : 3.9). Our present study reveals that SN35N-derived EPS consists of glucose, galactose, and mannose moieties at a ratio of 15.0 : 5.7 : 1.0. Thus, the molar ratios of the composed monosaccharides are significantly different, even among the same species.

It has been reported that EPSs produced by the *Lb. amylovorus* PY45 and *P. pentosaceus* LY45 inhibit the catalytic activity of hyaluronidase.[22] The phenomenon correlates with the inhibition of histamine release in inflammatory reactions accompanied by the immunoglobulin E-mediated mast cell degranulation.[44,45] The present study shows that the IC_{50} value for the SN35N-derived acidic EPS to hyaluronidase has almost the same as those of sodium cromoglicate and dipotassium glycyrrhizinate used as an anti-inflammatory agent (hyaluronidase inhibitors).[45,46] Interestingly, although our previous report has showed that the neutral EPS produced by *P. pentosaceus* LY45 inhibits hyaluronidase activity more effectively than did the acidic EPS, including fucoidan.[22] The

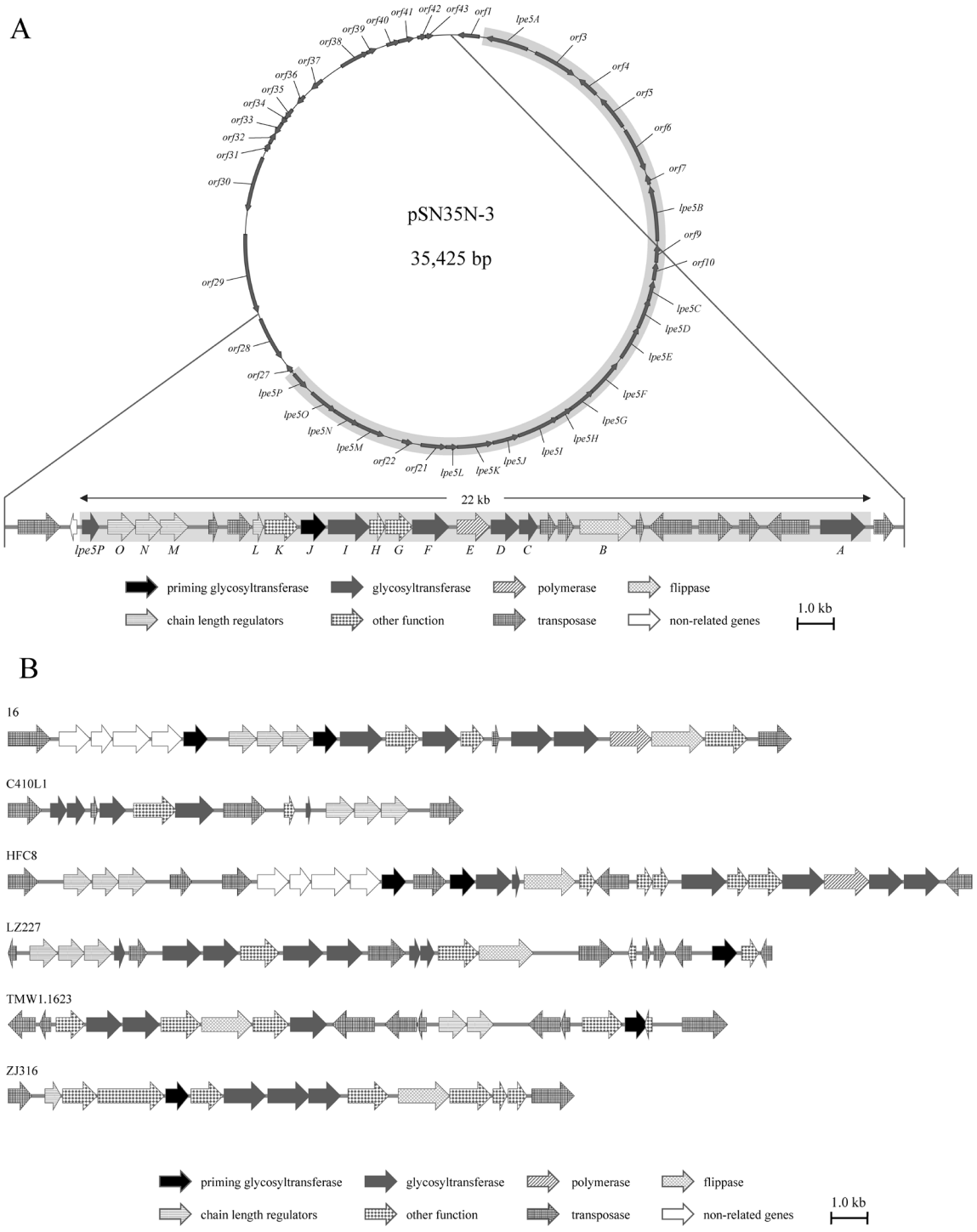

Fig. 4. Gene Organizations of Plasmid-Encoded EPS-Biosynthetic Gene Clusters

(A) Physical map and gene organization of plasmid pSN35N-3 (accession no. AP018408). Gene organization in putative EPS biosynthesis cluster *lpe5* is shown as a gray-shaded area. (B) The plasmid-encoded putative EPS biosynthesis gene clusters found on the *Lb. plantarum* strains are indicated with strain names as follows: strains 16 (accession no. NC_021514), C410L1 (NZ_CP017954), HFC8 (NZ_CP012650), LZ227 (NZ_CP015857), TMW1.1623 (NZ_CP017379), and ZJ316 (NC_020229).

IC_{50} value of the SN35N-derived acidic EPS is significantly lower than those of the acidic ones. In addition, the mixture of neutral and acidic EPSs may inhibit hyaluronidase activity synergistically. The synergistic inhibitory activity may be due to its structure or composition rather than its acidic properties.

A whole-genome analysis of the SN35N strain demonstrates that there are four possible EPS-biosynthetic gene clusters, designated *lpe1* to *lpe4*, on the chromosomal DNA (Table 3, Figs. 3, and 4). As compared with the *Lb. plantarum* WCFS1, although the *lpe1* cluster is not conserved, three other clusters are significantly conserved, especially in the *lpe4* cluster, against the cognate clusters designated *cps1–4* in WCFS1 (Fig. 3). Based on a homology search, the *lpe2* and *lpe4* clusters may be expected to function as intact gene clusters.

Although four EPS biosynthesis gene clusters are present on the *Lb. plantarum* SN35N chromosomal DNA, as shown in the result of the plasmid-curing experiment, it is suggested that the *lpe5* cluster found on the plasmid pSN35N-3 mainly

participates in the production of SN35N-derived acidic EPS. Furthermore, it has been shown that some *Lactobacillus* strains, such as *Lb. paracasei* DG,[47] *Lb. casei* YIT9018,[48] and *Lb. rhamnosus* RW-9595M,[49] have four genes, designated *rmlA–D*, which are necessary for synthesis of rhamnose as a component of the EPS in their EPS-biosynthetic gene clusters. In fact, these three strains produce EPS that contains rhamnose. However, *rmlA–D* genes are between the *lpe1* and *lpe2* clusters found on SN35N chromosomal DNA, whereas rhamnose was not detected as a component of the EPS from the SN35N strain. The results indicate that at least *rmlA–D* genes do not contribute to EPS production by *Lb. plantarum* SN35N. Our present results suggest that acidic EPS produced by the SN35N strain is phosphorylated EPS. However, a gene for the phosphorylation of EPS is not found near or in the EPS-biosynthesizing gene cluster.

Remus *et al.* investigated the deletion mutants of each *cps* gene cluster on *Lb. plantarum* WCFS1[50] and showed that two of the four *cps* clusters contain all of the genes required for polysaccharide formation (*cps2A–J* and *cps4A–J*). They demonstrated that all *cps* clusters function in the WCFS1 strain, despite of the incompleteness of other *cps1* and *cps3* clusters. This result shows the possibility that the existence of WCFS1 chromosome-encoded *lpe1–4* clusters is somehow connected to the structure and component sugars of the WCFS1-produced EPS.

In the present study, the EPS-biosynthesizing gene cluster found in the SN35N strain was compared with those in the WCFS1, SFA35B, and Lp90 strains (Fig. 3). The gene disruption experiment has suggested that ORFs for the biosynthesis of EPS encoded in the WCSF1 strain are present in the deduced EPS-synthesizing genes, designated *cps1*, *cps2*, *cps3*, and *cps4*.[50] In the case of the SF2A35B and Lp90 strains, it has been shown that the production of EPS was scarcely detected when the *cps2*-like gene was disrupted.[43] As shown in the present study, although the *lpe2–lpe4* clusters homologous to the *cps2–cps4* clusters are found on the SN35N genome, no EPS productivity was detected when the plasmid carrying the *lps5* gene cluster was cured from the SN35N cell by treatment with novobiocin. This result suggests that the EPS-biosynthesizing gene cluster is strain specific, even in the same *Lactobacillus* species. That is, even if several EPS-biosynthesizing gene clusters are found in *Lb. plantarum* strains, the EPS gene cluster for EPS production is strain specific.

Lb. buchneri CD034 and *Lactococcus lactis* NIZO B20, harboring plasmids designated pCD034-3[51] and pNZ4000,[52] respectively, have been reported to carry the EPS-biosynthesizing gene cluster, such as a *Lb. plantarum* SN35N plasmid pSN35N-3. The NCBI database suggests that six strains of *Lb. plantarum* may harbor the plasmid carrying the putative EPS-biosynthesizing gene cluster (Fig. 4B). However, studies confirming the phenomenon have not existed until now.

Interestingly, several transposase-encoding genes are found on the *lpe5* gene cluster located on the *Lb. plantarum* SN35N plasmid, pSN35N-3. At one time, the EPS-synthesizing gene cluster present chromosomally may have been responsible for the transposable integration into pSN35N-3. Table 3 shows that some *lpe5* cluster-encoded genes are homologous with that found in the genera *Leuconostoc* and *Bacillus*, whereas the *lpe1*, *lpe2*, *ple3*, and *lpe4* ones are homologous only with the species *Lb. plantarum*. This result indicates that the *lpe5* gene cluster is actively involved in the production of SN35N-specific EPS.

In recent years, the health benefits of natural polysaccharides, such as fucoidan and β-glucan, have drawn considerable attention.[53,54] Furthermore, as with other LAB species, there are some reports of a few strains of *Lb. plantarum* producing functional EPSs, such as radical scavengers, dendritic cell maturation induction, and anti-bacterial activities.[55–57]

The investigation of physiological functions of the SN35N-derived EPS, such as anti-allergy, anti-*helicobacter pylori*, anti-ulcer, and anti-viral activities, is in progress.

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Conflict of Interest The authors declare no conflict of interest.

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