Short communication: Genomic and phenotypic analyses of exopolysaccharides produced by *Streptococcus thermophilus* KLDS SM

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ABSTRACT

Streptococcus thermophilus plays important roles in the dairy industry. *Streptococcus thermophilus* KLDS SM could produce a high amount of exopolysaccharides (EPS). To understand the possible link between the genotype and the phenotype regarding EPS, the complete genome of *S. thermophilus* KLDS SM was sequenced and investigated in silico for genes related to carbohydrate fermentation, nucleotide sugars synthesis, and EPS gene cluster. We found that *S. thermophilus* KLDS SM is able to ferment sucrose, mannose, glucose, galactose, and lactose from the genomic research, which was confirmed by API 50 CH (bioMérieux, Marcy l'Etoile, France). The genetic analysis of nucleotide sugars and EPS cluster revealed that the EPS produced by this strain are composed of galactose and glucose, in accordance with the biochemical result. Furthermore, differences in the molecular mass of EPS from *S. thermophilus* KLDS SM cultivated under different carbon sources were correlated with the transcription levels of the genes encoding chain length determination protein and glycosyltransferase. Our findings provide a better understanding of the link between the genetic elements and the chemical conformation of EPS and a theoretical basis for producing tailor-made EPS through genetic and metabolic engineering approaches.

Key words: *Streptococcus thermophilus*, genome sequence, monosaccharide composition, molecular mass

Short Communication

Streptococcus thermophilus is a well-known, non-pathogenic lactic acid bacterium (LAB). Combined with *Lactobacillus delbrueckii* ssp. *bulgaricus*, it is traditionally used as a starter for fermented dairy food

products, such as yogurt, cheese, and ice cream (Liu et al., 2009; Settachaimongkon et al., 2014). Some previous studies suggested that *S. thermophilus* plays a vital role in treating lactose intolerance, antioxidant production, stimulation of intestinal immune responses, and relieving some cancers (Iyer et al., 2010).

Several *S. thermophilus* strains can produce exopolysaccharides (EPS), which can improve rheological and sensory attributes of fermented food products (Caggianiello et al., 2016). Moreover, some EPS from LAB have been shown to possess probiotic properties, such as antioxidant and antitumoral activities, reducing cholesterol, regulating the immune system, and providing substrates for the intestinal flora, which are related to the chemical conformation of EPS (Caggianiello et al., 2016; Cui et al., 2017). However, few reports offer scientific confirmation of the link between the genetic elements and the chemical conformation of EPS.

Streptococcus thermophilus KLDS SM was isolated from naturally fermented yogurt in Inner Mongolia, and it was shown to produce high levels of EPS (B. Li, F. Liu, and G. Huo, unpublished data). Based on this characteristic, *S. thermophilus* KLDS SM was selected to further clarify the possible link between the genotype and the phenotype regarding EPS. We analyzed the genetic elements involved in EPS biosynthesis to determine the structure of EPS and the transcriptional level of EPS-related genes under different carbon sources.

The genomic DNA of *S. thermophilus* KLDS SM was extracted by the DNeasy Tissue kit (Qiagen, Hilden, Germany). The whole-genome sequencing was performed using a combined strategy of Illumina HiSeq 2500 sequencing (insert size of 500 bp; Illumina, San Diego, CA) and Pacific Biosciences (PacBio) RSII sequencing (20,000 bp template library; PacBio, Menlo Park, CA) technologies. A total of 63,855 subreads and a total of 401 Mb of clean pair-end reads were obtained, respectively. Subsequently, PacBio RSII reads were de novo assembled into a circular contig with an average genomic coverage of 240 folds using the RS hierarchical genome assembly progress protocol (Chin et al., 2013).

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Pair-end reads were then used to correct the single base errors of PacBio RSII reads by SOAPsnv v1.05 (Li et al., 2009). Genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline (<http://www.ncbi.nlm.nih.gov/books/NBK174280>). The circular genomic map was constructed using CGView Server (Grant and Stothard, 2008). Functional annotation of protein-coding genes (**CDS**) was performed with the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) database (<http://www.genome.jp/kegg/>). Family distribution, conserved domain, and model structure of glycosyltransferase (**GT**) were analyzed using the carbohydrate-active enzymes database (<http://www.cazy.org/>), Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/cdd/?term=>), and SWISS-MODEL server (<https://swissmodel.expasy.org/>), respectively.

The profiles of carbohydrate fermentation were analyzed by API 50 CH test strip and API CHL medium (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Glucose, lactose, or sucrose was used in the chemically defined medium (Letort and Juillard, 2001) as the only carbon source at the final concentration of 1% (wt/vol). The EPS samples from chemically defined medium were extracted and then purified by DEAE-Sepharose Fast Flow (GE Healthcare, Pittsburgh, PA) and Sepharose CL-6B (GE Healthcare) following the method described by Ren et al., (2016). The monosaccharide composition and the molecular mass distribution of EPS were performed using high-performance anion exchange chromatography (ICS-3000, Dionex, Sunnyvale, CA) and high-performance size-exclusion chromatography (1260, Agilent, Santa Clara, CA) as previously described (Shao et al., 2014). The RNA isolation, reverse transcription, and real-time quantitative PCR (**RT-qPCR**) were implemented according to a previous protocol (Li et al., 2016). The specific primers used for the RT-qPCR are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13534>).

The genome of *S. thermophilus* KLDS SM consists of a circular chromosome (1,856,787 bp) with G + C content of 39.08% (Figure 1A). A total of 1,950 genes were predicted, including 1,732 CDS, 129 pseudogenes, 18 rRNA genes, 67 transfer RNA genes, and 4 noncoding RNA genes (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13534>). As shown in Figure 1B, 1,046 CDS were annotated into KEGG database; moreover, the highest number of 150 genes in the functional group associated with carbohydrate metabolism was found in the genome, suggesting that *S. thermophilus* KLDS SM may have advantage in carbohydrate utilization and EPS biosynthesis. Additionally, the genome neighbor report (Supplemental Table S3; <https://doi.org/10.3168/jds.2017-13534>) revealed that *S. thermophilus*

KLDS SM had 99.5673% symmetric identity with *S. thermophilus* ASCC 1275, which was reported to give a high EPS yield (Zisu and Shah, 2003).

Exopolysaccharide biosynthetic pathway involves the sugar uptake system, nucleotide sugar synthesis, polysaccharide synthesis, and export of the EPS (Laws et al., 2001; Cui et al., 2017). Genetic elements with EPS biosynthetic pathway were mined in the genome of *S. thermophilus* KLDS SM from these aspects. The most efficient sugar transport is the phosphoenolpyruvate-phosphotransferase system (**PTS**), which is composed of histidine-containing phosphoprotein, phosphoenolpyruvate-dependent phosphotransferase and sugar-specific permease enzyme (Laws et al., 2001). The genome of *S. thermophilus* KLDS SM harbors the histidine-containing phosphoprotein (A9497_02385) and phosphoenolpyruvate-dependent phosphotransferase (A9497_02380); in addition, genes responsible for sucrose and mannose PTS transporter sugar-specific permease enzyme were found in the genome (Supplemental Table S4; <https://doi.org/10.3168/jds.2017-13534>). Thus, we suggested that sucrose and mannose are the only 2 sugars that may be transported by PTS. Genes encoding lactose/galactose permease (A9497_02960) and glucose permease (A9497_00655) were identified in the genome. The ATP-binding cassette (**ABC**) transporter is not available to transport sugars into the cytoplasm of *S. thermophilus* KLDS SM, because it only equips 2 genes encoding ABC transporter (Supplemental Table S4). We found that *S. thermophilus* KLDS SM is able to use sucrose, mannose, glucose, galactose, and lactose from the genomic analysis. Carbohydrate fermentation results from the API 50 CH galleries also showed that *S. thermophilus* KLDS SM could metabolize these 5 sugars.

Once sugars are transported into the cytoplasm, they will be metabolized to the nucleotide sugars by various pathways, which act as active precursors to contribute to the EPS structure. As shown in Figure 2A, *S. thermophilus* SM could hydrolyze lactose into glucose and galactose by β -galactosidase (A9497_02955), then galactose is converted to the glucose-1-phosphate and the uridine diphosphate (**UDP**)-galactose the by the Leloir pathway (A9497_02965, A9497_02980, and A9497_02975). Glucose is phosphorylated by glucokinase (A9497_00055) to glucose-6-phosphate, which is further mutated to the glucose-1-phosphate by phosphoglucosylmutase (A9497_00345). Glucose-1-phosphate, as the vital intermediate product, could be transformed to the UDP-glucose by UDP-glucose pyrophosphorylase (A9497_05025) or to the deoxythymidine diphosphate (**dTDP**)-rhamnose using a series of enzymes encoded by A9497_02280, A9497_02270, A9497_02275, A9497_09195, and A9497_03320.

A

B

Figure 1. Circular genome map of *Streptococcus thermophilus* KLDS SM (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories in the complete genome (B). From outside to inside, circles (1, 2) show the locations of genes, including protein-coding genes (CDS), rRNA, transfer RNA (tRNA), and other genes on positive and negative chains. Circles (3, 4) show GC content and GC skew ($(G - C)/(G + C)$), respectively. Color version available online.

Figure 2. Bioinformatics analysis of genetic elements related to the exopolysaccharides (EPS) from the genome of *Streptococcus thermophilus* KLDS SM. (A) Pathways of nucleotide sugars synthesis, where locus tags (A9497_...) refer to the enzymes involved in the pathways, dotted arrows correspond to enzymatic reactions from inactive pathways, and red fonts present nucleotide sugars. (B) The EPS gene cluster, where functions and colors are marked below the gene cluster. Intact genes and pseudogenes are depicted by pentagons and chevrons, respectively. The locus tags (A9497_...) and lengths (bp) were marked above and below the genes. (C) Three-dimensional structure models of glycosyltransferase (GT). PTS = phosphotransferase system; GDP = guanine diphosphate; UDP = uridine diphosphate; dTDP = deoxythymidine diphosphate.

Three gene copies (A9497_01825, A9497_02805, and A9497_02970) encoding UDP-glucose 4-epimerase are characterized in the genome of *S. thermophilus* SM, which may endow it with the flexibility of the conversion between UDP-glucose and UDP-galactose. Sucrose and mannose are transported into the cytoplasm by the PTS and phosphorylated to sucrose-6-phosphate and mannose-6-phosphate, respectively, which are then catalyzed to fructose-6-phosphate. Sub-

sequently, fructose-6-phosphate is converted to UDP-*N*-acetyl-D-glucosamine by several enzymes encoded by A9497_00710, A9497_02310, and A9497_08950. We concluded that *S. thermophilus* SM can form 4 nucleotide sugars via in silico analysis, namely UDP-glucose, dTDP-rhamnose, UDP-galactose, and UDP-*N*-acetyl-D-glucosamine. These nucleotide sugars could be assembled into a repeating unit to construct EPS by the EPS gene cluster.

An EPS gene cluster (20,970 bp) was identified in the genome, consisting of 21 genes (A9497_01480 to A9497_01580; Figure 2B). In this EPS gene cluster, A9497_01580 to A9497_01565 are highly conserved genes assigned to biosynthesis regulation and chain length determination of EPS. The gene A9497_01560 encodes the priming GT, which involves in transferring the sugar to a lipid carrier as the first step in the synthesis of EPS. Both A9497_01525 and A9497_01530 may have the same function as A9497_01565 and A9497_01570, which determine the chain length of EPS, but they were assigned as pseudogenes. Pseudogene A9497_01510 and A9497_01515 encode flippases, which are associated with the export of repeating units. In the gene cluster, A9497_01500 and A9497_01550 encoding transposases were attributed to pseudogenes, indicating that they are not capable of transposition. Gene A9497_01495 encodes *orf14.9*, which is associated with the cell growth of *S. thermophilus* (Wu et al., 2014). The genes A9497_01490, A9497_01485, and A9497_01480 were assigned as phosphatase (pseudogene), phosphoglycerate mutase, and permease. At the 5' end of the EPS gene cluster, we detected a gene (A9497_01590) encoding purine-nucleoside phosphorylase, which is also present in other EPS gene clusters (Wu et al., 2014).

Furthermore, the genes (A9497_01520, A9497_01535, A9497_01545, and A9497_01555) were responsible for different GT. The substrate specificity prediction for GT in the EPS gene cluster will provide a wealth of information on the monosaccharide composition of the EPS produced by *S. thermophilus* KLDS SM. The genes A9497_01520, A9497_01535, A9497_01545, and A9497_01555 were identified as 1–4 galactosyltransferase, glucosyltransferase, glycosyltransferase, and α -12-L-rhamnosyltransferase (truncated) by sequence similarity analysis, respectively. Genes A9497_01520 and A9497_01535 were classified to the GT-2 family, however, A9497_01545 is not associated with any carbohydrate-active enzymes families. Thus, it seems important to perform manual annotation of this gene. First, the conserved domain of A9497_01545 was analyzed by searching the Conserved Domain database, the result showed that it only possessed the domain of unknown function (DUF4422) without typical domains of GT. Subsequently, we predicted the 3-dimensional structure of A9497_01545 using the SWISS-MODEL server, its 6 models from 16 templates were shown in Figure 2 C; no models were fitted with the topology of GT-A, GT-B, and GT-C proteins. Based on these analyses, A9497_01545 did not encode GT and was tentatively curated as EPS biosynthesis protein. Bioinformatics analysis above showed that *S. thermophilus* KLDS SM can also synthesize dTDP-rhamnose and UDP-*N*-ace-

tyl-D-glucosamine, but it does not own the functional genes involved in corresponding GT in the EPS gene cluster. Thus, we speculated that the monosaccharide composition of EPS produced by *S. thermophilus* SM is without rhamnose or UDP-*N*-acetyl-D-glucosamine.

To understand the possible link between the genotype and the phenotype about EPS, the EPS samples obtained by the supplement of glucose (**EPS-G**), lactose (**EPS-L**), or sucrose (**EPS-S**) were purified by 2 steps, as shown in Figures 3A and 3B. The elution profile of each EPS showed only a single symmetrical peak, suggesting they were homogeneous polysaccharides. After 48 h of fermentation, the optimal EPS yield (96.33 mg/L) in purified form was found in the supplement of sucrose (Figure 3C). A similar result was obtained by Li et al., (2016), probably because sucrose can be taken up via PTS as mentioned in the genomic data, which is the most efficient sugar transport system. The glycosyl compositional results revealed that EPS-G, EPS-L, and EPS-S were composed of glucose and galactose (Table 1), which was in agreement with the analysis *in silico*. The monosaccharide composition of these EPS was also accordance with the EPS produced by *S. thermophilus* 05–34 (Li et al., 2016) and *S. thermophilus* Cth-9204 (Pachekrepapol et al., 2017). Moreover, no difference in the molar ratio of galactose to glucose (1.8:1) in the 3 types of EPS was observed (Table 1). According to the calibration curve of standards, the molecular masses of EPS-G, EPS-L, and EPS-S were estimated to be 1.26×10^5 , 4.22×10^5 , and 1.51×10^6 Da (Table 1), respectively. Our results agree with those in *Lactobacillus fermentum* TDS030603 and *Lactobacillus rhamnosus* E/N, which is an EPS produced on different carbohydrate sources with the same monosaccharide composition but different molecular mass distributions (Fukuda et al., 2010; Polak-Berecka et al., 2015).

As the difference in molecular mass may be determined by polymerization degree and GT, which are related to A9497_01570 encoding a putative chain length determination protein, A9497_01520 encoding a putative galactosyltransferase, and A9497_01535 encoding a putative glucosyltransferase, the expression profile of these 3 genes was determined following incubation of *S. thermophilus* KLDS SM in the presence of glucose (control) or sucrose using RT-qPCR. As shown in Figure 3D, when the CDS was supplemented with sucrose, the transcription levels of the genes A9497_01570, A9497_01520, and A9497_01535 were upregulated by 3.2, 2.1, and 2.1 fold, respectively, indicating that these 3 genes are upregulated in response to the increase of the molecular mass of EPS by increasing chain-length. Interestingly, the same upregulation folds of A9497_01520 and A9497_01535 were in line with the same monosaccharide composition (galactose and

Figure 3. Elution profile of exopolysaccharides (EPS) on DEAE-Sepharose Fast Flow column (A) and elution profile of EPS on Sepharose CL-6B column (B; both GE Healthcare, Pittsburgh, PA) and the effect of carbon sources on the EPS production (C) and the effect of carbon sources on the relative expression of 3 EPS-related genes (D). EPS-G, EPS-L, and EPS-S represent the EPS obtained by *Streptococcus thermophilus* KLDS SM cultivated under the supplement of glucose, lactose, and sucrose, respectively. Expression of each gene under the supplement of glucose was arbitrarily assigned as 1. Error bars represent standard deviation of 3 independent measurements. * $P < 0.05$.

glucose) in the EPS-G and EPS-S, reconfirming that A9497_01520 and A9497_01535 encoded GT responsible for galactose and glucose.

Although *S. thermophilus* was highly conserved in carbon source utilization, including the PTS and ABC transport system, the EPS gene cluster is another key

factor for the production and the chemical conformation of EPS. Liu et al. (2009) reported that EPS biosynthesis genes of *S. thermophilus* are either obtained by strain-specific horizontal gene transfer events or may have evolved much faster than other genes. This implied that EPS gene clusters of *S. thermophilus* have different gene compositions and contexts and share limited sequence similarities. The sequence of the EPS gene cluster of *S. thermophilus* KLDS SM was aligned (Megablast; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) against the NCBI nucleotide collection. The results indicated that the EPS gene cluster of *S. thermophilus* KLDS SM had 99% nucleotide identity to that in *S. thermophilus* ASCC 1275, which earlier demonstrated high EPS production and has 4 genes for determining EPS chain length (Zisu and Shah, 2003; Wu et al., 2014). Interestingly, the corresponding ho-

Table 1. The monosaccharide composition and molecular mass of exopolysaccharides (EPS) produced by *Streptococcus thermophilus* KLDS SM under different carbohydrate sources¹

Sample	Molar ratio (galactose/glucose)	Molecular mass (Da)
EPS-G	1.8 ± 0.02	1.26 × 10 ⁵
EPS-L	1.8 ± 0.05	4.22 × 10 ⁵
EPS-S	1.8 ± 0.03	1.51 × 10 ⁶

¹EPS-G, EPS-L, and EPS-S represent the EPS obtained by *S. thermophilus* KLDS SM cultivated under the supplement of glucose, lactose, and sucrose, respectively. Values are reported as means ± SD (n = 3).

mologous sequences were also found in the genome of *S. thermophilus* KLDS SM. These observations informed our proposition that *S. thermophilus* KLDS SM is potentially a high EPS-producing strain. In addition, EPS yield may be determined by the transcription level of related genes, which will be studied in our future work.

Most studies focused on the relationship between EPS gene cluster and EPS production (Wu et al., 2014; Bai et al., 2016), whereas few focused on the link between the genetic elements and the chemical conformation of EPS. Our results indicated that the phenotypic profiles of carbohydrate utilization, nucleotide sugars synthesis, and monosaccharide composition of EPS were determined by the genetic basis and the molecular mass of EPS was resulted from the transcription levels of the genes encoding chain length determination protein and GT. A combined investigation of genomics and phenotypic traits could be an efficient strategy for understanding the gene structure relationships of EPS and this study will help to exploit the EPS with defined composition.

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