**Isolation of an exopolysaccharide-producing *Streptococcus thermophilus* from
Algerian raw cow milk**

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1
2 **Abstract**

3 Lactic acid bacteria (LAB) play a key role in the development of the organoleptic and
4 textural qualities of fermented dairy products. The exopolysaccharides (EPS) produced
5 by LAB are of great technological interest since they improve the rheological
6 characteristics of these products and help reduce syneresis. In the present work, an EPS-
7 producing *Streptococcus thermophilus* strain was isolated from Algerian raw cow
8 milk. Its identity was confirmed by biochemical and molecular methods. The presence
9 in its genome of a *priming glycosyltransferase* gene was confirmed by PCR. The
10 strain's growth and EPS production in milk was analysed and the molar mass of the
11 EPS produced determined by HPLC.

12
13 **Key words:** *Streptococcus thermophilus*, Exopolysaccharide, Priming-
14 glycosyltransferase gene, EPS production in milk, Molar mass.

1 **Introduction**

2

3 The lactic acid bacteria (LAB) are Gram-positive microorganisms that play an essential

4 role in the industrial production of fermented dairy products. Their enzymatic activities

5 and the metabolic products they generate during fermentation and ripening confer the

6 rheological and organoleptic qualities desired upon these products. Some strains are

7 known to synthesize exopolysaccharides (EPS). These macromolecules play an

8 important role in the development of the texture of yoghurt and other fermented milks,

9 cheeses and low fat dairy desserts [1, 2]. EPS can be classified into two groups:

10 homopolysaccharides (HoPS) and heteropolysaccharides (HePS) [2, 3]. HoPs are

11 composed of monosaccharide subunits, while HePS are formed from a backbone of

12 repeated subunits of different monosaccharides, e.g., D-galactose, D-glucose or L-

13 rhamnose, in different ratios. Different types of linkage can occur within a repeating

14 unit, determining the rigidity and rheological properties of a HeP [4]. Well known

15 examples of HoPS include the dextrans and fructans produced by *Leuconostoc*

16 *mesenteroides* and *Streptococcus salivarius* respectively [3], while HePS are

17 synthesized by many LAB including strains of *Streptococcus thermophilus*,

18 *Lactococcus lactis* and a number of dairy lactobacilli [5]. The total yield of EPS

19 depends on the composition of the growth medium (carbon and nitrogen sources,

20 growth factors, etc.), the temperature, pH, and the incubation time. Whereas mesophilic

21 strains can produce maximal amounts of HePS under suboptimal growth conditions,

22 production by most of the studied thermophilic LAB strains is growth-dependent [3].

23 The quantity of EPS produced by LAB in milk varies widely among species and indeed

24 strains. In some cases, only small amounts are produced in the food matrix, and even

1 this production is commonly unstable [5]. This instability has been related to the loss of
2 plasmids coding for the EPS gene cluster.

3 The *Qualified Presumption of Safety* (QPS) or *Generally Recognized As Safe* (GRAS)
4 status of many LAB renders them very attractive as EPS producers for the food industry
5 [5], and important advances have been made in the study of EPS production by these
6 bacteria in recent years. The increasing demand for fat-free or reduced fat products is
7 also raising interest in the use of EPS-producing LAB as natural bio-thickeners.

8 *S. thermophilus* is a LAB widely used in the manufacture of dairy products -in fact it is
9 the most important thermophilic dairy starter [6, 7, 8]. The species is able to grow (or at
10 least survive) at the high temperatures (45°C) required in various dairy product
11 manufacturing processes. For cheese-making, *S. thermophilus* is used alone or in
12 combination with different lactobacillus and mesophilic starters, while for yoghurt it is
13 normally used with *Lactobacillus delbrueckii* subsp. *bulgaricus* [9]. The role of *S.*
14 *thermophilus* in milk fermentation is the rapid conversion of lactose into lactic acid,
15 which causes a rapid reduction in the pH and encourages the production of metabolites
16 important to the final flavour [7]. A number of *S. thermophilus* strains synthesise EPS
17 [10, 11, 12], which contribute to the desirable viscous texture and rheological properties
18 of fermented milk products, particularly yoghurt [10, 13].

19 The interest of industry in finding starters with new metabolic properties has revived the
20 idea of screening artisanal dairy products of different origin. Currently, much attention
21 is being paid to improving the texture of food by screening for new EPS-producing
22 strains. The aim of the present work was to isolate EPS-producing *S. thermophilus*
23 strains from raw cow milk samples from Algeria, and to characterise the EPSs they
24 make.

Materials and Methods

Bacteria and growth conditions

Candidate EPS-producing bacterial strains were isolated from raw milk samples by serial dilution in peptone saline solution [peptone (1 g/L) and NaCl (8.5 g/L)] followed by streaking on the surface of M17 agar supplemented with 5.0 g/L of lactose (LM17) (Oxoid, UK). These plates were incubated at 42°C for 48 h under anaerobic conditions, established using Anaerocult A tablets (Merck, Germany), to isolate thermophilic microorganisms.

Escherichia coli used for cloning procedures were grown in Luria medium (LB) [14] at 37°C; when appropriate ampicillin (100 mg/L) was added. For long-term maintenance, stock cultures were stored in 20% (v/v) glycerol, 80% (v/v) LM17 or LB at – 80°C.

Exopolysaccharide production

Possible EPS-producing strains were identified via their mucoid or ropy appearance. EPS production was assessed by Indian ink (Art. Material S.A., China) staining, and by growing the candidate strains on ruthenium red (Riedel de Haën AG, Germany) agar (RRM17). Reuthenium red (0.08% wt/vol) was added to LM17 as described by Dabour and LaPointe [15]. This stain tints the bacterial cell wall: non-EPS-producing strains produce red colonies while EPS-producers are white.

Identification of isolated strains

The isolated candidate strains were identified by phenotypic and molecular methods. Colony morphology, Gram staining, catalase reaction, tolerance to 4% (w/v) and 6.5% (w/v) NaCl, growth at 10 and 45°C, and the hydrolysis of starch and esculin were all

1 examined. Carbohydrate fermentation assays were also performed in a specially
2 designed M17 broth without glucose but supplemented with 5.0 g/L each of lactose,
3 inulin, sorbitol, mannitol, galactose, salicin, cellobiose, maltose, melibiose and raffinose
4 (Biochemika, UK). Bromocresol purple was added (0.004%) as a pH indicator. The
5 cultures were incubated at 42°C and sugar fermentation determined at 24 h and 48 h.
6 Carbohydrate fermentation was shown by the change in the colour of the medium from
7 purple to yellow. Analysis of the results classified all candidate EPS-producing strains
8 as belonging to the genus *Streptococcus*. The inability to ferment maltose was used to
9 distinguish *S. thermophilus* from the other streptococci [16].

10 To confirm the identity of the selected EPS-producing *S. thermophilus* strains,
11 molecular methods were used. PCR amplification of the *16s rRNA* gene was performed
12 with pA/pH primers [17] in a MyCyclerTM (Bio-Rad, USA) thermocycler using the
13 conditions described by Edwards et al. [17]. PCR products were purified using the GFX
14 PCR DNA Gel Band Purification Kit (GE Healthcare, UK). The nucleotide sequence
15 was obtained using an ABI Prism 373 automated sequencer (performed by Secugen,
16 Spain) and compared with DNA sequences held in the National Center for
17 Biotechnology Information (NCBI) database using the Basic Local Alignment Search
18 Tool (BLAST, <http://www.ncbi.nlm.nih.gov/BLAST>).

19 20 *Detection and sequencing of the priming glycosyltransferase gene*

21 Total DNA was isolated as described by de Vos and Simons [18] and plasmid isolation
22 according to de Vos et al. [19]. Detection of the *priming glycosyltransferase (GT)* gene
23 was performed by PCR using total DNA as a template under the conditions described
24 by Provencher et al. [20]. The PCR product was cloned into the pGEM-T Easy Vector
25 (Promega, Spain) according to the vector manufacturer's protocol.

Electrotransformation of *E. coli* was undertaken in a Bio-Rad pulser apparatus (Bio-Rad, USA) following the manufacturer's recommendations. Plasmids from *E. coli* were isolated by the alkaline lysis method [14]. Restriction endonuclease (Takara, Japan) digestions were performed according to the recommendations of the enzyme manufacturers, and DNA analysis of the fragments performed as described by Sambrook and Russell [14]. DNA sequencing was performed as described above. DNA hybridisations were performed using the non-radioactive DNA Labelling and Detection Kit (Roche Molecular Biochemicals, Germany), following the manufacturer's instructions.

Fermentation conditions and establishment of EPS-producing S. thermophilous cultures in milk

Commercial milk powder (Candia, Algeria) was reconstituted to 10% (w/v) in distilled water and heated for 5 min at 95°C. Different flasks were then inoculated with a 1% overnight culture of the EPS-producing *S. thermophilous* strain raised in LM17 broth, and incubated in a water bath at 37°C for 16 h, or at 42°C for 6 h or 12 h. The acidification of these cultures was measured using a pH210 pH meter (Hanna Instruments, France). Serial dilutions of the cultures were made in peptone before plating on LM17 agar (performed in triplicate). Viable counts were performed after incubation at 42°C for 48 h.

Isolation and quantification of EPS

The EPS fraction was isolated from the fermented milk after 6 h and 12 h of incubation at 42°C or after 16 h at 37°C, according to the protocol of Salazar et al. [21]. Briefly, 40 g of fermented milk were mixed with a trichloroacetic acid (TCA) solution (12% final

concentration) and stirred vigorously for 45 min at room temperature. Bacteria and proteins were removed by centrifugation ($10,000 \times g$, 4°C for 30 min.) and the pH of the supernatant increased to 4.5 ± 0.5 using a 10 N NaOH solution. EPS was precipitated by adding two volumes of cold ethanol (95%) to the supernatant; the mixture was then continuously stirred for 48 h at 4°C . After centrifugation as described above, the EPS fraction was resuspended in ultrapure water and intensively dialysed (three water changes every 24 h) against ultrapure water for 3 days at 4°C using successive dialysis membranes (Sigma, UK) with molecular weight cut-offs of 6-8 and 12-14 kDa. The obtained EPS was then freeze-dried. All EPS isolation was performed in duplicate. The amounts of EPS produced were initially evaluated gravimetrically by measuring the polymer dry mass (PDM) after 48 h of drying at 42°C .

Quantification of EPS and molar mass determination

The molar mass (MM) of the EPS fractions was determined by size-exclusion chromatography (SEC) using an HPLC system running Empower software and equipped with an Alliance 2690 module injector, a PDA 996 photodiode array detector, and a 410 refractive index (RI) detector (Waters, USA). Freeze-dried fractions of the dialysis product (see above) were resuspended (5 mg/mL final concentration) in 0.1 M NaNO_3 , maintained overnight under gentle stirring, and centrifuged at $10,000 \times g$ for 10 min before analysis. Separations were performed in a TSK-Gel G3000 PWxL and TSK-Gel G5000 PWxL column placed in series, protected by a TSK-Gel guard column (Supelco-Sigma, USA). The injection sample size was 50 μL and the mobile phase was 0.1 M NaNO_3 . Separations were performed at 40°C and at a flux rate of 0.45 mL/min. The EPS peaks were detected by their RI; the presence of proteins that might interfere with the RI analysis was monitored using the PDA detector at 280 nm. Dextran

standards (Fluka-Sigma, USA) ranging from 5×10^3 to 4.9×10^6 Da were used to determine the MM of the EPS. The regression equations for determining the quantity of EPS were calculated using four different concentrations from (0-1 mg/mL) of each of seven dextran standards ($R^2 \geq 0.98$). The regression equation for the determination of MM was calculated using the elution times of the seven dextran standards ($R^2 = 0.99$).

Results and Discussion

Screening for EPS-producing Streptococcus thermophilus strains

Fifteen raw cow milk samples from different farms in the Mostaganem region of Algeria were used to screen for EPS-producing *S. thermophilus* strains on LM17 agar at 42°C. Of approximately 1000 candidate colonies, 20 were originated by bacteria with a mucoid - though not necessarily EPS producing - phenotype. [22]. Although, it has been reported that the use of non-milk-based media is not the most suitable for screening for EPS-producing strains [5]; since the number of EPS isolates could be underestimated, it is much easier to use growth media than milk for isolation purposes in the laboratory. These 20 mucoid isolates were selected for further analysis.

Identification of EPS-producing strains

The 20 selected isolates were initially examined using simple taxonomic tests such as Gram-staining, cell morphology analysis, and the catalase assay. All were classified as LAB cocci growing in chains; all were able to grow at 45°C but not at 10°C. The capacity to grow at different NaCl concentrations and to hydrolyse esculin was tested to differentiate streptococci from enterococci. Four strains were classified as streptococci based on their inability to hydrolyse esculin and their lack of growth at either 4% or 6.5% (w/v) NaCl. The sugar fermentation profiles (mainly the inability to use maltose)

of these four strains identified them as belonging to *S. thermophilus* [23]. The analysis of the *16S rRNA* gene sequence confirmed this identity (>99% sequence similarity).

Although the four colonies had been selected for their mucoid appearance on LM17 agar, only two of them were white on RRM17 plates, suggesting only these were able to produce EPS. This was further confirmed in broth; the strain producing the highest EPS yield (data not shown) was then selected for further analysis. This was designated as *S. thermophilus* BN1.

As shown in Fig. 1, the India ink staining technique showed the *S. thermophilus* BN1 cells to be linked in chains surrounded by a transparent halo. This halo denoted the existence of an organized structure, similar to a capsule, surrounding the cells. The presence of capsular EPS has been described in some *S. thermophilus* strains [2, 24], although the capsules involved are usually thicker than that of *S. thermophilus* BN1 (see Fig. 1 for dimensions).

Detection of priming glycosyltransferase genes

The first step in the assembly of the repeating unit in HePS synthesis is achieved by priming GT. The genes encoding priming GT are well conserved. Provencher et al. [20] described hybrid primers G-*-Bact-a-F-36 and G-*-Bact-a-R-27 for the detection of the *priming GT* gene in *S. thermophilus*. In the present work, a PCR fragment of the expected size (260 bp) was obtained when chromosomal DNA was used as the template. This amplicon was cloned and sequenced, and showed strong similarity to the *priming GT* genes of other *S. thermophilus* EPS clusters deposited in the NCBI database. The sequence obtained was deposited in the GenBank database under accession number Hx2000001349. The alignment of the translated amino acidic

sequence showed that, although well conserved, the deduced protein was different to the priming GT of other bacteria (Fig. 2).

Location of the *priming GT* gene in the *S. thermophilus* genome

All the EPS clusters described to date in *S. thermophilus* appear to be chromosomally encoded [10]. In contrast, in most of the mesophilic LAB strains, exopolysaccharide production genes are encoded by plasmid DNA [3]. Plasmid isolation was attempted in the present *S. thermophilus* BN1 using standard procedures, but none were obtained. In fact, plasmids have been reported present in only 20-30% of *S. thermophilus* strains [25]. In addition, most of those described have been under 10 kb in length [25], while EPS clusters are usually encoded by genomic sequences over 20 kb in length [10].

The 0.26 kb-long internal fragment of the *priming GT* gene was used as a probe to locate the gene in the *S. thermophilus* genome. Hybridisation fragments of different size were detected when the total DNA was digested with *Bam*HI, *Bgl*II, *Cla*I and *Hind*III endonucleases (8, 4, 5 and 7 kb respectively; Fig. 3). Two bands of 5.0 and 1.5 kb were detected after *Eco*RI digestion, which is consistent with the *Eco*RI site located in the 0.26 kb fragment used as a probe.

The results indicate that the *priming GT* gene, and therefore the EPS cluster, is located in the chromosome of *S. thermophilus* BN1. This is interesting from a technological viewpoint; the localization of the EPS cluster in plasmids would be associated with the instability of the EPS-producing phenotype.

Growth and acidifying activity of *S. thermophilus* BN1 in milk

S. thermophilus BN1 was inoculated into milk at an initial concentration of 1% (v/v) and incubated at either 37°C or 42°C. The strain produced the largest number of viable

cells after 12 h of incubation at 42°C, or after 16 h at 37°C (1.20×10^9 CFU/mL and 2.25×10^9 CFU/mL respectively). After 6 h of incubation at 42°C, the strain showed a slightly lower number of viable cells (5.30×10^8 CFU/mL) but enough for milk curdling to occur; the pH fell during the incubation period, reaching final values of 5.03 ± 0.04 and 4.66 ± 0.01 after 6 h and 12 h of fermentation at 42°C respectively, and 4.60 ± 0.01 after 16 h at 37°C. *S. thermophilus* BN1 may therefore be considered a strain with a good capacity for growth in milk and to have acceptable curdling abilities.

Quantification of EPS production and determination of the EPS molecular mass

The gravimetric method was initially used to determine the amount of EPS produced, which under all conditions was around 300 mg PDM/L. No significant differences were observed between the quantities of EPS produced after 6 h and 12 h at 42°C or after 16 h at 37°C. The quantification of EPS production in *S. thermophilus* based on the measurement of PDM has been reported by several authors [11,12], with different values obtained depending of the use of a culture medium, milk or enriched milk. Most of the studied strains showed EPS yields in milk below 100 mg PDM/L; only a few have been reported to produce up to 150 mg PDM/L [12]. Thus, the amount of EPS produced by *S. thermophilus* BN1 in milk was higher than that reported for other EPS-producing *S. thermophilus* strains under similar conditions. When the EPS produced was analysed by Sephadex exchange chromatography (SEC), the values returned were between 5 and 10 times lower (Table 1). This is not surprising since other compounds such as glucomannans, proteins and sugars are co-purified with the EPS and can make up to 94% of that measured in PDM analysis [12], depending on the culture medium and purification method used [26]. The quantitative results returned by HPLC indicate

the optimal conditions of EPS production to be 16 h at 37°C (Table 1). In thermophilic bacteria, the production of EPS is usually associated with growth [3].

SEC analysis revealed the existence of two EPS peaks with different MM (7.2×10^5 and 1.4×10^4 Da) under all study conditions (Table 1). The production of two types of EPS is known in some bacteria [27], including *S. thermophilus* strains [12] in which two types of EPS with different MMs has been described. However, the fact that only one priming GT was found in *S. thermophilus* BN1 suggests that these peaks might represent two fractions of the same EPS. The production of EPS types of different MM might be influenced by culture conditions, the available carbon and nitrogen sources, and the carbon/nitrogen ratio [26]. However, with respect to the present representatives of *S. thermophilus* BN1, neither increasing incubation time nor the temperature affected the MM of the peaks (Table 1). Neither was the proportion of the two EPS types affected (data not shown). It has been suggested that the production of more than one EPS fraction by a single strain might be due to the partial hydrolysis of a starting polymer by intracellular enzymes released over time into the medium [28]. In *S. thermophilus* BN1, however, prolonged fermentation times did not affect the MM of the EPS polymer (Table 1).

In this work, an EPS-producing strain of *S. thermophilus* was isolated that might be used as a starter in the production of dairy products. Further attempts should be made to find new EPS-producing strains in different raw materials and artisanal products; in this way the number and diversity of strains with the potential to be used as starters could be increased.

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3

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1

2 **Fig. 1:** Morphology of the isolated strain as observed after staining with India ink. The
3 arrow indicates the presence of the capsule-like EPS.

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Fig. 2

Multiple alignment of the partial *priming GT* sequences of *S. thermophilus* BN1, *S. thermophilus* (AY057915.1), *S. thermophilus* CNRZ1066, (NC_006449), *S. thermophilus* LMD9 (NC_008532.1), *Streptococcus pneumoniae* SP18 (NZ_ABAE01000002.1) and *Leuconostoc mesenteroides* (DQ873505.1). Conserved amino acids in all sequences are shaded black; similar residues in some are shaded gray.

<i>S. thermophilus</i> BN1	---FHFIRKTSIDELPQFWNVLKGDMSLVGTRPPTLDEYESYTPEQKRRLSEKPGITGLWLVTGRNNIN	66
<i>S. thermophilus</i>	TKIGGILRKTSIDELPQLINVFLGOMSLVGPRPLPDREIIEYGDNDKFSLVKPGMTGWWQVSGRSTIG	107
<i>S. thermophilus</i> CNRZ1066	TKIGGILRKTSIDELPQLINVFLGOMSLVGPRPLPDREIIEYGDNDKFSLVKPGMTGWWQVSGRSTIG	107
<i>S. thermophilus</i> LMD9	---GHFIRKTSIDELPQFWNVLKGDMSLVGTRPPTLDEYESYTPEQKRRLSEKPGITGLWQVSGRSEIT	70
<i>S. pneumoniae</i> SP18	---GHFIRKTSIDELPQFYNVLKGDMSLVGTRPPTVDEYEHYTPEQKRRLSEKPGITGLWQVSGRSEIK	70
<i>Lc. mesenteroides</i>	----HFIRKTSIDELPQFWNTLKGTMSIIGPRPVVEDELVEYGEDIDKFSLVKPGVFGLWQISGRNNIK	66

Fig. 3

Total DNA from *S. thermophilus* BN1 (A) was digested with *Bam*HI (1), *Bgl*II (2), *Cla*I (3), *Eco*RI (4) and *Hind*III (5). (B) Southern blot analysis using an internal fragment of the *priming GT* gene. MW: GeneRuler™ DNA Ladder Mix (Fermentas, Lithuania).

Table 1: Growth characteristics of *S. thermophilus* BN1 and EPS production in milk at 37°C and 42°C.

Growth temperature		37°C		42°C	
Incubation time		16 h		6 h	12 h
Viable cells (CFU/mL)		2.25×10 ⁹		5.30×10 ⁸	1.2×10 ⁹
pH		4.6±0.01		5.03±0.04	4.66±0.01
EPS production	HPLC (mg/L)	58.9±0.1		48.3±0.1	36.9±0.3
Number of EPS peaks		2		2	2
EPS MM		7.2×10 ⁵	1.4×10 ⁴	7.2×10 ⁵	1.4×10 ⁴
		7.0×10 ⁵		1.3×10 ⁴	