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Comparison of the Expression Levels of Exopolysaccharide Genes in *Streptococcus thermophilus* LBB.T54 Grown in Semi-Defined Media and Milk

Miroslava Terzieva* and Zoltan Urshev

LB Bulgaricum PLC, R&D Center, 12A Malashevska str., Sofia, Bulgaria

Abstract

Exopolysaccharide (EPS) production by *Streptococcus thermophilus* strains is exploited by dairy companies to obtain fermented milk with improved texture. In previous studies of *S. thermophilus* LBB. T54, however, EPS yields in M17 were negligible compared to milk. It remained unclear whether differences in the composition of the medium simply stimulated the growth and viable cell counts or also changed the expression level of the *eps* cluster. The aim of the study was to test the potential change of the expression levels of selected genes from the *eps* cluster in *S. thermophilus* LBB.T54 when grown in M17, compared to milk. Strain LBB.T54 was cultured in LM17 (M17 with lactose) or milk at 37°C. qPCR was performed with primer pairs targeting the *eps*D, *eps*E and *rpo*A genes. The *rpo*A gene was used to normalize the results. qPCR measurements clearly indicated a 2-4 fold higher expression levels of both *eps*D and *eps*E in strain *S. thermophilus* LBB.T54 when grown in milk compared to LM17. Growth in other semi-defined media (M17 with glucose, Elliker broth and modified Trypticase soy broth with glucose) resulted in similar expression levels of *eps*D and *eps*E as that in LM17. EPS yields in milk reached 33 mg/l while in LM17 they did not exceed 5 mg/l. In the process of starter culture preparation and from technological point of view, the application of LM17 medium or another semi-defined medium seems to be counter-productive compared to milk-based media when EPS yields are essential for products obtained with LBB.T54.
Keywords: exopolysaccharide, expression, M17, milk, qPCR, *Streptococcus thermophilus*

Резюме

Образуването на извънклетъчни полизахариди (ИПЗ) при щамове *Streptococcus thermophilus* се използва от млекопреработвателите за получаване на ферментирали млека с подобрена структура. В предишни изследвания на *S. thermophilus* LBB.T54, обаче, добивите от ИПЗ получени в M17 бяха пренебрежимо малки в сравнение с млякото. Остана неясно дали разликите в състава на хранителните среди просто стимулират развитието и броя на живите бактерии или променят също и нивата на експресия на полизахаридния (*eps*) клъстер. Целта на изследването беше да се определят потенциалните промени в нивата на експресия на избрани гени от *eps*-клъстера при култивирането на *S. thermophilus* LBB.T54 на среда M17 и на мляко. Щам LBB.T54 беше култивиран на LM17 (M17 с лактоза) или мляко при 37°C. Беше проведен количествен PCR (qPCR) с праймери, насочени към гените *eps*D, *eps*E и *rpo*A, като *rpo*A генът беше използван за нормализиране на резултатите. qPCR-измерванията ясно показаха 2-4-кратно по-високи нива на експресия на *eps*D и *eps*E при развитието на *S. thermophilus* LBB.T54 на мляко в сравнение с LM17. Култивирането на щама на други полу-дефинирани среди (M17 с глюкоза, Еликер бульон или модифициран триптиказен бульон с глюкоза) показаха сходни нива на експресия на *eps*D и *eps*E като тези на LM17. Добивите на ИПЗ на мляко достигаха 33 mg/l докато на LM17 те не надхвърляха 5 mg/l. От технологична гледна точка в процеса на приготвяне на закваска, използването на LM17 или друга полу-дефинирана среда изглежда контра-продуктивно в сравнение с млечна среда, когато добивите на ИПЗ са от значение за продукти, получени с LBB.T54.

* *Correspondence to: Miroslava Terzieva*
E-mail: m.terzieva@lbbulgaricum.bg

Introduction

Exopolysaccharide-producing strains of lactic acid bacteria are applied by dairy producers to modify the texture of fermented milk – to increase viscosity, creaminess and stability (De Vuyst *et al*., 2003).

In *Streptococcus thermophilus,* EPS synthesis is proportional to biomass accumulation and therefore optimizing the growth conditions results in improved polymer yields (De Vuyst *et al*., 1998). EPS yields are increased with the addition of low molecular nitrogen sources such as casein hydrolysate, yeast extract or peptones (Cerning *et al.*, 1990; García-Garibay and Marshall, 1991; Sebastiani and Zelger, 1998; Ricciardi *et al*., 2002). However, little is known about the regulation at gene level of the *eps* cluster in this species.

In *S. thermophilus,* the *eps* genes are chromosomally located in a succession of up to 12 or 13 open reading frames (Stingele *et al*., 1996; Almirón-Roig *et al*., 2000), which are transcribed in a single mRNA. The *eps* gene cluster starts with more constant sequences of regulatory genes, followed by variable in content and order genes, encoding glycosyltransferases, that make the chemical composition and structure of the EPS unique to a particular strain. *eps*D and *eps*E are known to be present in most strains of *S. thermophilus* as they are related to the regulation and the initial step in EPS synthesis, respectively. A recent review on the genetics and application of EPS in lactic acid bacteria was published by Zeidan *et al*. (2017).

For qPCR and gene expression analysis, a careful selection of a house-keeping reference gene is essential. For *S. themophilus* the *rpo*A gene was selected from a list of previously validated reference genes (Sumby *et al*., 2012; Løvdal and Saha, 2014).

Strain *S. thermophilus* LBB.T54 was found to produce a neutral EPS with molecular mass of 1,6 x 10^6 Da composed of glucose:galactose:rhamnose ratio of 5:1:1 (Urshev *et al*., 2008)

The aim of this study was to develop a suitable qPCR method to test the potential change of the expression levels of selected genes from the *eps* cluster in *S. thermophilus* LBB.T54 when grown in M17, compared to milk.

Materials and Methods

Bacterial strains and growth conditions

Strain *S. thermophilus* LBB.T54 is an industrial strain maintained in the LBB Culture collection (LB Bulgaricum PLC, Sofia, Bulgaria) selected for its ability to produce viscosity in milk. LBB.T54 was cultured in LM17 (M17 with lactose) (Terzaghi and Sandine, 1975) or sterile 10% reconstituted skim milk. Additionally, other semi-defined media were used – GM17 (M17 with glucose), Elliker broth or modified Trypticase soy broth with glucose. All incubations were performed overnight at 37°C.

RNA isolation

Two milliliters of culture were used to isolate RNA with the E.Z.N.A. Bacterial RNA Kit (Omega Bio-tec, Norcross, USA). For the milk cultures one additional preliminary step was added and milk was solubilized with the addition of 3 volumes of 2% sodium citrate – 0,5N NaOH solution, followed by centrifugation at 5000 g for 5 min. All next steps were performed according to the manufacturer's instructions. The quantity and purity of RNA were assessed spectrophotometrically. The quality of RNA was confirmed electrophoretically to visualize intact 16S- and 23S-rRNA bands.

qPCR reaction

Quantitative PCR (qPCR) was performed using the iTaq™ Universal SYBR® Green One-step Kit (Bio-Rad Laboratories, Hercules, CA, USA) on a C1000 Touch Thermal Cycler appended with CFX Real-Time System (Bio-Rad Laboratories). The qPCR reactions (10 microliters) contained

Table 1. Primers used to perform qPCR in the study

Name	sequence, 5'-3'	target
epsD186F	TGTTGGGCTCCGAACACTTC	internal region within
epsD435R	ACGACTACGAGCAACTTCCATCAA	*eps*D
epsE892F	GCTCAAGACCGTGTGGGAGAA	internal region within
epsE1237R	TTCGACCGCTTACTTGCCAAA	*eps*E
rpoA468F	TGCACCAGTGGGAACTTTGG	internal region within
rpoA625R	CGAGGGCATCTTCTGGGATG	*rpo*A

40ng of each RNA preparation and 10 pmol of the primer pairs listed in Table 1. No-template controls and no-reverse transcriptase controls were also included. The amplification conditions recommended by the manufacturer of the kit were used. Expression levels were calculated with the Comparative C_T method ($\Delta\Delta C_T$) using the expression level in LM17 as baseline (1.00).

EPS quantification

EPS was quantified using a previously described method (Urshev *et al*., 2008) Briefly, 50 ml of sample were treated with 17,5% (v/v) 80% Trichloroacetic acid and centrifuged at 10000 g for 20 min. Three volumes of cold absolute ethanol were added to the supernatant and EPS was precipitated overnight at 4°C. EPS was then pelleted by centrifugation at 6000 g for 20 min and resuspended in 20 ml hot water, dialyzed for 48h against distilled water and quantified spectrophotometrically with the phenol-sulfuric acid method (Dubois *et al*., 1956). Glucose was used as standard and results were presented as mg/l glucose equivalents. The values obtained for non-inoculated media were subtracted from the result obtained for each sample.

Results

All preparations obtained with the E.Z.N.A. kit contained a typical yield of 40 ng/µl RNA with A260/A280 ratio within 1,8-2,0 and visible intact 16S- and 23S-rRNA bands after electrophoretic separation. Control qPCR reactions without reverse transcriptase (noRT-controls) showed difference in Cq values greater than 6 compared to RT-samples, indicating that residual DNA was not interfering in the analysis.

Triplicate qPCR measurements in two independent trials clearly indicated a 2-4 fold higher expression levels of both *eps*D and *eps*E in strain *S. thermophilus* LBB.T54 when grown in milk compared to LM17 (Fig. 1). Good correlation was observed between the expression levels of these two genes as they are co-expressed as members of the same cluster. Growth in other semi-defined media resulted in similar expression levels of *eps*D and *eps*E as that in LM17 (results not shown).

The averaged EPS yields for the two independent trials reached 33 mg/l in milk while in LM17 they did not exceed 5 mg/l.

Discussion

In previous studies of *S. thermophilus* LBB.T54, EPS yields in M17 were negligible compared

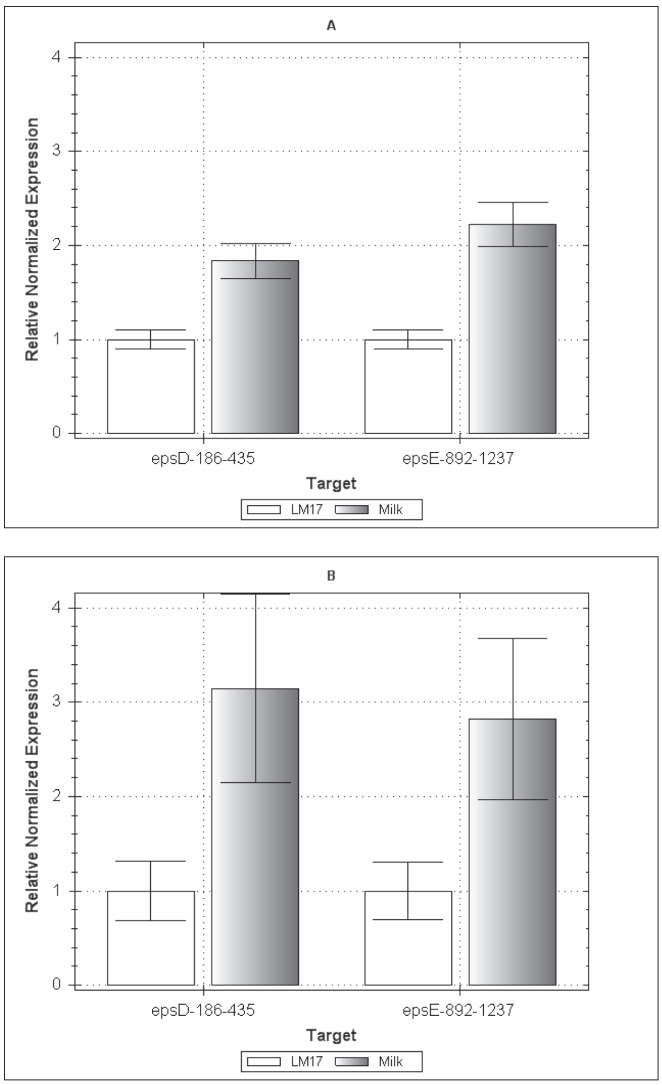

Fig. 1. Relative expression levels of the *eps*D and *eps*E genes of *S. thermophilus* LBB.T54 grown in LM17 and milk medium. Results from two independent trials (A and B) are presented. Error bars are derived from triplicate measurements.

to milk while the addition of low molecular nitrogen source, such as yeast extract, to milk improved EPS yield (Urshev, 2009). It remained unclear whether differences in the composition of the medium simply stimulated the growth and viable cell counts or also changed the expression level of the *eps* cluster.

The obtained results in the present study showed that compared to semi-defined media such as M17, milk not only offered much richer nutritive environment for EPS synthesis, but also stimulated the expression of the genes from the *eps* cluster. Also the difference in expression levels in the two media indicates that the *eps* cluster in this strain is tightly regulated. Other authors demonstrated that the expression of genes encoding enzymes related to sugar nucleotide synthesis, precursors of the EPS polymer, strongly influenced EPS yields (Escalante *et al*., 1998; Degeest and de Vuyst, 2000; Svensson

et al., 2005). In addition, we have found that in *S. thermophilus* LBB.T54 gene cluster of EPS synthesis itself has different expression levels that correlate to EPS yields.

The two genes *eps*D and *eps*E belong to a single cluster and are transcribed in a single mRNA. Our results showed a good correlation of the expression levels of these two genes. We can therefore assume that targeting of either gene for performing the qPCR analysis gives adequate picture of the expression of the whole *eps* cluster.

The qPCR method developed in this study proved to be suitable to assess the expression of the *eps*D and *eps*E genes form the *eps* cluster of *S. thermophilus* LBB.T54 in a manner that can predict the EPS yield. Lower expression levels of the two *eps* genes, when using LM17 as growth medium, correlated with low EPS yields. Therefore, experimental data obtained with qPCR can contribute to further optimization of growth conditions to achieve higher and constant EPS yields and improved texture of fermented milk using *S. thermophilus* LBB.T54 as starter culture.

Conclusion

From technological point of view, the application of LM17 medium or another semi-defined medium for starter culture preparation seems to be counter-productive compared to milk-based media when EPS yields are essential for products obtained with *S. thermophilus* LBB.T54. Monitoring the expression level of the *eps* cluster in this strain can give further insight to the effect of different stress factors that are part of the starter culture process such as freezing and freeze-drying in order to avoid loss and improve the EPS-producing capacity of the strain.

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