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A REVIEW ON MOLECULAR DETECTION OF *STREPTOCOCCUS THERMOPHILUS* IN DAIRY PRODUCTS

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Abstract:

Streptococcus thermophilus is one of the commercially important dairy bacterium and considered as second most important starter culture in the manufacturing of dairy products (Yoghurt and cheese). To enhance the variety of these starter culture are not only essential to bring new strains with unique and desired characteristics, but also for the maintenance of these yoghurt starters which reduces with the overuse. In this review, different genes are used for the detection of S. thermophilus by molecular methods and as well strains producing exopolysaccharide are discussed. Molecular analysis including pulsed field gel electrophoresis and polymerase chain reaction are addressed. Since the dairy industry is keep in searching of new dairy starters for increasing the diversity of its product range, nowadays their keen attention is to explore new starter strains with high potentiality to give fermentation products.

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INTRODUCTION:

The genus *Streptococcus* consist of numerous harmful pathogenic species e.g. *S. pneumonia* and *S. pyogenes*, including this one of the Generally Recognized As Safe species, *Streptococcus thermophilus*. The most important feature of the *S. thermophilus* that it is harmless in nature as a food microorganisms and it is also used as a starter culture in the manufacturing of dairy products [1-3] with annual market value of B$40 billion. Significantly, annual range of consumption is about 1021 live cells by human population [3]. As this bacterium is widely used in the dairy industry for the manufacturing of yoghurt, but for this purpose traditionally they shows symbiotic relationship with *Lactobacillus delbrueckii* subsp. *bulgaricus* and this association among them through which they gain benefits from each other is called proto cooperation [4]. By this relation they often show a high acidification rate [5-10] with low pH [11, 12] and high yield of *S. thermophilus* population [11-13] motivate high production of exopolysaccharide (EPS) [14] compared to monoculture and upgraded stability of the final product [15]. This cooperation is highly industrial interest because they influence a large fermentation product. So far, evidence that *S. thermophilus* require each of the species associated with them for the exchange of nutrition [16]. In terms of nutritional exchange this relation have highly very important but in global level it remains poorly documented. Various approaches have been taken in emerging isolation and identification measures for this bacteria; a) can be normally separated into two sets: the use of biochemical characteristics somewhat specific to strains *Streptococcus thermophilus* b) genes detection such as *lacZ*, *epsC* etc. The purpose of this review is to describe molecular techniques for the detection of *S. thermophilus* and selection of the *S. thermophilus* strains producing (EPS) in dairy products.

History and evolutionary origin of *Streptococcus thermophilus*

Streptococcus thermophilus is the type of lactic acid bacteria [17]. In 1919 Orla-Jensen was firstly recognized that *S. thermophilus* to the *streptococci* group of lactic acid bacteria [18]. Basically *S. thermophilus* having following characteristics such as: Homo-fermentative, gram positive, facultative anaerobic, catalase, oxidase and cytochrome negative and as non-spore forming and non-motile. Morphological characteristics show that *S. thermophilus* has ovoid-spherical in shape with the range of diameter is about 0.7 to 0.9 µm and occurs in chains and in pairs. The optimum temperature for the growth of this bacterium is about 40-45 °C, gives minimum growth at 20 to 25°C and maximum growth at 47 to 50 °C [17]. *S. thermophilus*may tolerate high rate of temperature for 30 minutes [19] like upto 60 °C. The fermentation capacity of *S. thermophilus* shows varies among different sugars such as; it can ferment lactose, sucrose, glucose and fructose but unable to use arginine. Galactose utilization of *S. thermophilus* species are very rare. Lactic acid isomer like L (+) lactic acid is the predominant production of *S. thermophilus* and through fermentation of lactose there is high production of lactic acid, diacetyl and acetaldehyde.

Although, *S. thermophilus* shows similarities in its genotyping and phenotyping characteristics with other lactic acid bacteria, but in systematic grouping still it is not comfort. In previous *S. thermophilus* was find to be the sub species of *S. salivarius* [20]; but in recent homological study of DNA-DNA of *S. thermophilus* reclassified as a species of separate group of streptococcus. One of the unique characteristic of *S. thermophilus* among other streptococci is that, it does not have an antigen of N group. The DNA ranges between guanine and cytosine is about 37.2-40.3% [21]. Few of the strains of *S. thermophilus* having the ability to produce exopolysaccharides and for the enhancement of growth require some amino acids like histidine, cysteine, valine, leucine, isoleucine, methionine arginine, tyrosine tryptophan and glutamic acid as well. A type of peptidoglycan Lys-Ala [19] is present in their cell wall and due to its high heat resistance to the temperature and in association with one or more species of lacto bacillus it is mostly used in the production of yoghurt and some different types of cheeses e.g. Italian and Swiss type cheeses. *S. thermophilus* highly present in dairy products and the isolation of *S. thermophilus* is possible from the dairy products by providing optimum incubation temperature such as; >40 °C in this temperature the bacteria could be possible to isolate from the raw milk. In recent year the positive identification of suspect colonies have been made possible through DNA-DNA probes. Commonly in the regeneration section of the plate exchanger pasteurizer is the conjoint place for *S. thermophilus* where they grows as biofilm [17].

Streptococcus thermophilus genome

The size of the genome of *S. thermophilus* is very small than its nearest relative species of lactic acid bacteria. In *S. thermophilus* an average of 1.75-1.85 Mb and A054, respectively, vs 2.35 Mb of Lactococcus lactic. In Gene-Bank until now more than 100 DNA sequences entries have been reported to be listed. After a serial sub culturing on solid media *S. thermophilus* may show genetic instability. Pebay *et al.,* (1993) described few of the loci which represent instability or revealing sequence polymorphism in *S. thermophilus*. The morphological features of *S. thermophilus* may affected by its genetic instability [22]. Pebay *et al.,* (1993) found four variants of *S. thermophilus*

CNRZ368, which were different in shape, size and opacity. In *S. thermophilus*it is characterized that up to now five of the enzymes encoded chromosomally for restriction and modification [22]. Extensively it is reported to be studied genetic characterization of the *S. thermophilus* system of phosphoenolpyruvate (PEP) dependent phosphotransferase (PTS) [23], polysaccharide production [24], protein and peptide utilization [25], phage resistance mechanisms and the stress response system. Remarkably during the last period's basic understanding of biosynthesis and genetics of (EPS) producing *S. thermophilus* has been increased.

PCR-based detection and genotyping of *Streptococcus thermophilus*

Nowadays PCR amplification is consider as a one of the important process for the detection of bacteria. The reason behind this is that within one hour a single copy of DNA can be amplified in to several copies of the template DNA, which is rapid as compared to any other non- molecular methods. However, PCR gives false positive result too, because it can amplify dead cells, so the care must be taken doing the process. Moreover, this makes the analysis of data more complex and it is the problem that have to be highlighted as it has a future suggestion from an authorized perception.

PCR Components

For running PCR techniques numerous efforts are required, each having its own importance such as;
Buffer: During the reaction buffer provides buffering capacity and ionic strength to the reaction. Mainly, it assists the process of annealing through the cations which avoid the repulsive force between backbones of negatively charged (dsDNA) [26].

dNTP: dNTP are the deoxynuleotide tri phosphates. It act as a raw material along with the template and primer in the formation of amplicons. Each nucleotide is made up of 4 dNTPs, such as; dTTP, dCTP, dATP and dGTP [26] and primer are oligonucleotides (5-3) having the length of about 20 nucleotides [27].

PCR phase

PCR process consist of three phases are as follows:
a) Denaturation b) Annealing c) Elongation

a) Denaturation: By heating 94 ºC the double stranded DNA became single strands because the molecule holding hydrogen bonding disrupted and detached. By doing so, the primers access to the single strand of template (DNA) [26].

b) Annealing: By providing 50 ºC the reaction mixture becomes cooled in this phase and permitting the primer to select the appropriate position on the template DNA molecule (which target specific sequence of nucleotide that used to be amplify) to further hybridize the complementary strand to proceed [26].

c) Elongation: In this phase 72 ºC heated the reaction mixture, template DNA is read by heat stable polymerase (3′-5′ direction) and by using PCR components which are the building blocks in this reaction (free dNTP's and buffer) leads to form a new complementary strand of DNA (5′-3′) [26].

This cycle repeat and the target is doubled, yield 1 million copies of DNA from the fragment target DNA, after completing of 30 cycles, which makes the DNA easy to detect by gel electrophoresis process [28].

Pulsed Field Gel Electrophoresis (PFGE)

It is a technique in which entire genome proceed by restriction enzymes, which forms a macro restriction profiles distinct to one or more isolates by splitting the bacterial genome irregularly, and producing small numbers of large DNA fragments. It assists departure of fragments (large fragments over 10 Mb) contrasting to Restriction Endonuclease Analysis (REA), detached only small fragment 30-50 kb [29].
PFGE must be handled by skilled person. It can also use to generate genetic data which can be analyzed statistically.

Identification by species-specific PCR and *lacZ* gene sequencing

Several ways of identification of *S. thermophilus* are found, but to overcome time consuming were replaced by molecular biological methods, such as;

a) Colmin *et al.,* (1991) published species-specific gene probes DNA fragment (4.2 kb) hybridizing to the closely related species *S. thermophilus* and *S. salivarius* and as well polymorphism of intraspecific restriction fragment [30].

b) Enermann *et al.,* (1992) also differentiate *S. salivarius* and *S. thermophilus* by a gene probes based on (23S rRNA sequence) [31].

c) Lick and Teuber *et al.,* (1992) derived an oligonucleotide probe which is also Species-specific for *S. thermophilus* [32].

For further differentiation on strain level of *S. thermophilus* accomplished by fingerprinting followed by the of restriction endo nuclease including pulsed field gel electrophoresis and ribotyping as well.
To enhance the sensitivity of detection, Lick *et al.,* (1996) developed a species-specific PCR amplification based on *lacZ* gene, this method is provide the way of accurate and rapid identification of *S. thermophilus*. For further confirmation a PCR product 968 bp in length, can be obtained by hybridization a specific

oligonucleotide gene probe of *S. thermophilus* [33] shown in table 1.

This PCR technique can be useful in the identification of *S. thermophilus* present in milk products like yoghurt. In this study Lick *et al.,* used (34 strains) of lactic acid bacteria including (lactobacilli, streptococci, and enterococci) [33] shown in table 2.

Table 1: Primers used for lacZ gene sequencing

Target gene	Primer sequence	Amplicon size
lacZ	F (5′-CACTATGCTCAGAATACA- 3′)	968 bp
	R (5′-CGAACAGCATTGATGTTA- 3′)	

Table 2: Strains investigated by primer specific PCR-amplification

No.	Species	Strain	Source	Species-specific amplification product
1	*Streptococcus thermophilus*	55a	BAM	+
2	*Streptococcus thermophilus*	71	BAM	+
3	*Streptococcus thermophilus*	910	BAM	+
4	*Streptococcus thermophilus*	J-31	BAM	+
5	*Streptococcus thermophilus*	St-22	BAM	+
6	*Streptococcus thermophilus*	ER-1	BAM	+
7	*Streptococcus thermophilus*	20617	DSM	+
8	*Streptococcus thermophilus*	St-13	BAM	+
9	*Streptococcus mutans*	68693	BAM/Hy	–
10	*Streptococcus salivarius*	20560	DSM	–
11	*Streptococcus salivarius*	7724	BAM	–
12	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Kt1	BAM	–
13	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Kt3	BAM	–
14	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Kt4	BAM	–
15	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Milsa	BAM	–
16	*Lactobacillus delbrueckii* subsp. *bulgaricus*	3041	BAM	–
17	*Lactobacillus delbrueckii* subsp. *delbrueckii*	20074	DSM	–
18	*Lactobacillus delbrueckii* subsp. *bulgaricus*	20081	DSM	–
19	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Vitus	BAM	–
20	*Lactobacillus delbrueckii* subsp. *delbrueckii*	3512	BAM	–
21	*Lactobacillus delbrueckii* subsp. *lactis*	19e	BAM	–
22	*Lactobacillus delbrueckii* subsp. *lactis*	20072	DSM	–
23	*Lactococcus lactis* subsp. *lactis*	C-2	BAM	–
24	*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*	Bu-1	BAM	–
25	*Lactococcus lactis* subsp. *cremoris*	F7/2	BAM	–
16	*Lactococcus lactis* subsp. *cremoris*	KS-11	BAM	–
27	*Lactobacillus acidophilus*	3062	BAM	–
28	*Lactobacillus brevis*	3028	BAM	–
29	*Lactobacillus fermentum*	3025/71	BAM	–
30	*Lactobacillus helveticus*	20075	DSM	–
31	*Lactobacillus plantarum*	3017	BAM	–
32	*Enterococcus faecalis*	DS-5	BAM/Hy	–
33	*Enterococcus faecalis*	20406	BAM	–
34	*Enterococcus faecium*	20477	BAM	–
35	*Escherichia coli*	HB101	BAM	–

Source: Lick *et al.,* 1996

Non-specific amplification was not seen, the product was only specific to target DNA sequencing of the *S. thermophilus*, even showed no amplification from closely related *S. salivarius*. This analysis, consequently may be useful for fast species-specific identification of *S. thermophilus* with no further need for hybridization [33].

Genotyping of *Streptococcus thermophilus* strains by sequencing *serB* gene

El-Sharoud *et al.,* (2012) have described the genotyping of *S. thermophilus* by analyzing the variations among the house keeping genes that encodes *serB* gene (phosphoserine phosphate). By analyzing 54 *S. thermophilus* isolates, it was found that the assay could successfully distinguished having diversity in *serB* alleles (Figure 1). These *S. thermophilus* isolates showed five different *denove* alleles of *serB* which could not found in any other *S. thermophilus*, present the database of multi locus sequence typing (MLST). Such analysis by El-sharoud *et al.,* based on nucleotide sequence of the *serB* gene gives the possible way of probing diverse genotype of *S. thermophilus* [34].

Table 3: Primers used for *serB* gene sequencing

Target gene	Primer sequence	Amplicon size
serB	F (5′-GGTCCAAGAAGAAGTAATTGA- 3′)	498 bp
	R (5′-GACCTTATACAAATCTGGTT- 3′)	

Source: El-Sharoud *et al.,* 2012

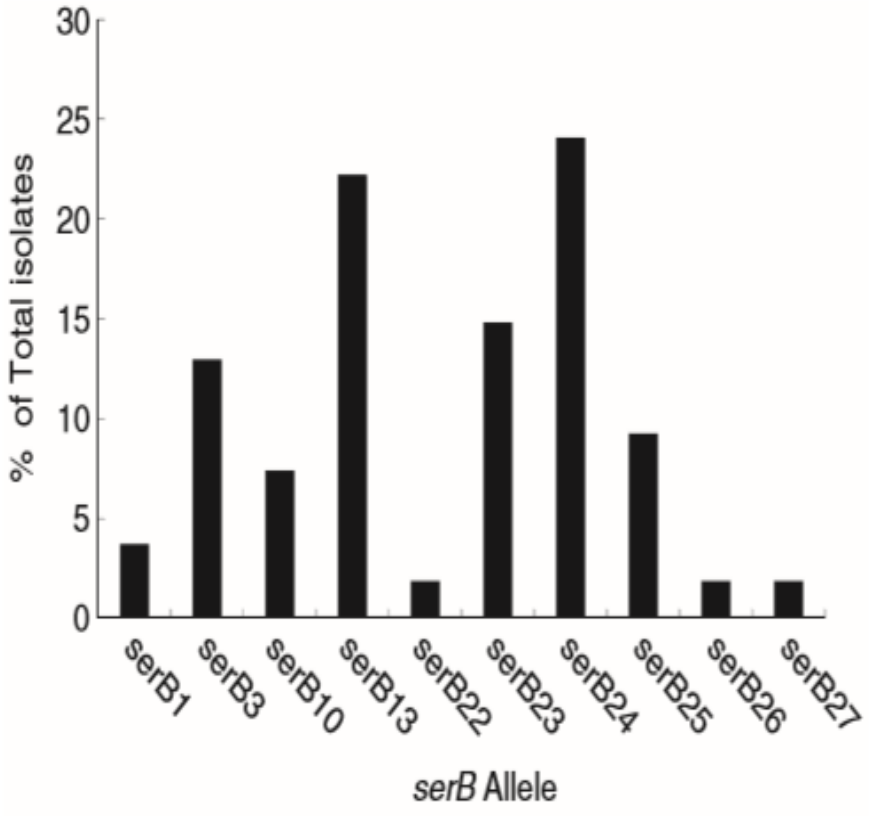

Fig 1: Distribution of *serB* alleles in *Streptococcus thermophilus*

PCR amplification and 16s rRNA sequencing

Erkus *et al.,* (2013) determined identification of *S. thermophilus* by species-specific PCR and sequenced 16s rRNA gene. By analyzing, 14 artisanal yoghurt sample 66 strains were successively identified as *S. thermophilus* and the genotyping among the diversity of strains also recognized by applying (PFGE); produced 22 homology groups. For this purpose firstly, the isolated strains were analyzed by species-specific PCR amplification based on an intragenic fragment (968 bp) within the *lacZ* gene of *S. thermophilus* [33]. The results of isolates showed all the same expected amplicon size. Then the verification of results were based on sequencing the 16s rRNA gene of one of the representative strain cTY17. After determination of 16s rRNA gene of 1460 bp was deposited in Gene-Bank with the accession number of (EU694138) and through the analysis of (PFGE) found 22 homology groups, each showed diversity among the restriction profile [35].

PCR-based detection of *Streptococcus thermophilus* strains producing exopolysaccharides (EPS)

Tidona *et al.,* (2016) worked on the selection of those *S. thermophilus* strains which are able to produce (EPS). They collected 139 strains of *S. thermophilus* in which some of the genes were leading to produce (EPS). They screened the genes by PCR technique and obtained 29 strains were PCR positive and they also inoculated the same strains in the skim milk to highlight their some other properties like ropiness. This is one of the property which makes the milk culture more thicken. Through this selection three strains were recognized as starter culture for dairy products with increasing rheological approach. For molecular screening of (EPS) genes four primer were used which sequences present in Gene-Bank: CP002340.1; pointing six of the operon regions [36].

Genotyping represented the presence and absence of four PCR outcome, assigning values of 1 and 0 respectively and phenotyping data were explained by multivariate analysis in principle components analysis (PCA). Viscosity measurement at different rotation speeds recorded both at upward and downward stream (Figure 2) [36].

Table 4: Primer sued for 16s rRNA

Target gene	Primer sequence (2 external primers and 2 inner primers)	Amplicon size
16S rRNA	F (5′-AGAGTTTGATCCTGGCTCAG- 3′)	1460 bp
	R (5′-AGGGTTGCGCTCGTTG- 3′)	
	F (5′-CTACGGCTACCTTGTTACCA- 3′)	
	R (5′-GTGCCAGCAGCCGCGGTAA- 3′)	

Source: Erkus *et al.,* 2013

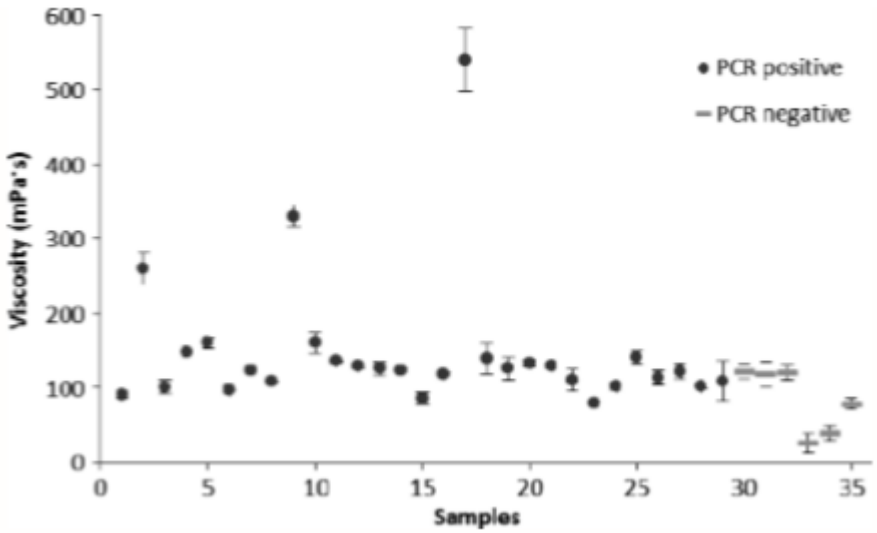

Fig 2: Strains selected as PCR positive and PCR negative for seeming viscosity and (EPS) genes

Table 5: Primers used for the selection of *S. thermophilus* producing (EPS)

Target gene	Primer sequence	Amplicon size
deoD-epsA	deoD F (5′ -GGTTACACAGGAACATACAAGGGTGAA- 3′)	2048 bp
	epsA R (5′CCAACCGGGAAATCAAACTTCTCTTT- 3′)	
epsC-epsD	epsC F (5′-AGTGATGAAATCGACGTACT- 3′)	1425 bp
	epsD R (5′-CCAACCGACTTTTCTACGAC- 3′)	
epsE	epsE F (5′-CCATCCTTCATTACTACTACTTTAATT- 3′)	1343 bp
	epsE R (5′-CGCATTGAAATTGGTATTATACAGT- 3′)	
cpsX	STACpsX F (5′-CAGTTCATTTGGTTGGTTGTGCTGTTT- 3′)	960 bp
	CpX R (5′-GAACCACCAACTACATAAGGATAGATGA- 3′)	

Source: Tidona *et al*., 2016

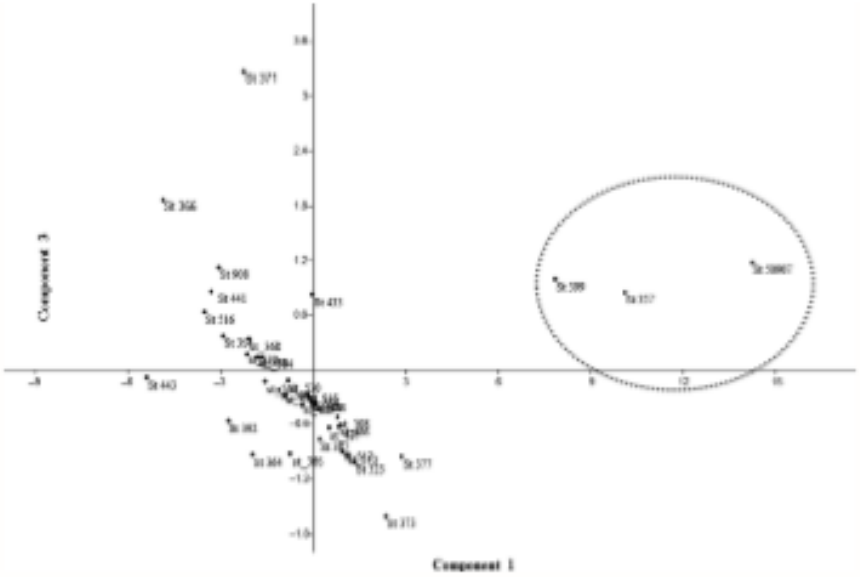

Fig 3: Scatter plot representing 29 PCR positive and 6 negative strains

For the determination of viscosity capacity Turkeys test applied. Phenotyping (PCA) of the data flow curves from the upward and downward, including the PCR data exposed define separated locus of the 3 ropy strains; St 399, St 357, and St 50907 (produced highly viscous culture) on the right side of the graph (Figure 3). Equally, on the left side of the graph 6 PCR negative strains were placed. Merging the genotyping and phenotyping results they found a successful way to detect *S. thermophilus* producing exopolysaccharides [36].

CONCLUSION:

In this review, we have deliberated the few genetic approaches which have been used to detect *S. thermophilus*. This microorganism demonstrated as safe, due to this nature extensively used in the manufacturing of yoghurt and cheese making and undoubtedly has been ever since humans initial adept in dairying [1]. While other detection methods such as conventional microbiological techniques remain an essential part but they are lengthy and time consuming. For that reason, further advance method should be made to improve the specificity and efficiency of molecular methods in order to increase and support the capability of numerous reference and regional laboratory in molecular techniques for the selection of technological important starter culture for the manufacturing of diverse dairy products.

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