

# Genome-wide analysis of a potent functional dairy starter bacterium *Streptococcus thermophilus* MTCC 5460: a comprehensive study of its dairy Niche adaptive features

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**Genomic analysis of *Streptococcus thermophilus* strain MTCC 5460, an isolate from market dahi (curd), revealed particular gene features that contributed towards its adaptation to a dairy-specific niche. The genome comprising 1.6 Mb, encoding 1809 genes, revealed the presence of genes involved in lactose/galactose utilization; well-developed proteolytic system including cell envelop proteinases and several transporters; and bacteriocin synthesis and competence proteins involved in defence mechanism, which help prevent food spoilage. The genome comprised genes for stress resistance property of the strain, contributing to its gut endurance and gene encoding formation of aroma compounds. Unlike pathogenic streptococci, genes for virulence property were absent in the genome. Overall, the study revealed features within the genome that enabled the organism to survive in a gastric environment and assisted in its interaction with the host microbiota and mucosa, thus, validating the strain as a potent functional dairy starter and a promising candidate for potential probiotic applications.**

**Keywords:** Dairy starter, genome, MTCC 5460, probiotics.

*STREPTOCOCCUS THERMOPHILUS* is a low G + C, Gram-positive, non-motile, non-spore-forming, catalase-negative, facultative anaerobe, homo-fermentative lactic acid bacterium<sup>1</sup>. Notably, *S. thermophilus* belongs to the thermophilic group and is extensively used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus* in the manufacturing of yogurts and cheese at a relatively high process temperature (45°C)<sup>2</sup>. It is considered as the second most important industrial dairy starter after *Lactococcus* (*Lc.*) *lactis*. Over the past few years, functional starter cultures have been highly explored for use in food fermentation indus-

tries to naturally achieve desired properties in food products. In addition to industrial applications, the species is also reported as a potential probiotic organism<sup>3</sup>. *S. thermophilus* have been associated with various health effects such as immune stimulation of host, production of pro-inflammatory cytokines<sup>4</sup>, prevention of chronic gastritis<sup>5</sup> and change in the composition of oral microbiota subjected with halitosis<sup>6</sup>. Nevertheless, the virulence gene present in other streptococci is completely absent or non-functional in *S. thermophilus*, indicating their evolutionary divergence or reduction due to adaptation in dairy-specific niche which favoured the safe position of the dairy *S. thermophilus* strains<sup>7</sup>.

Understanding of human genome led to address that each human being harbours genes other than their own, which belong to intestinal microbiota. Current research is focused on obtaining genomic data of intestinal microbiome and probiotic micro-organisms that impart health benefits. The genomic data provide information regarding the influence of gut commensal on human hosts by providing maturational signals for the developing infant and alteration in the barrier mechanisms of the gut<sup>8</sup>. Thus, *S. thermophilus* is an extremely important species in the dairy industry and its in-depth genomic insight will provide useful information for its preferred application.

To meet the demanding need of probiotic products, genome sequencing based in-depth analysis of *Lactobacillus* stains has gained lot of attention<sup>9,10</sup>. At the National Center for Biotechnology Information (NCBI) microbial genomes database, a total of 51 completed genome sequences of genus *Streptococcus* are listed. In this context, the present study was carried out with the aim of sequencing the *S. thermophilus* MTCC 5460 genome and performing comparative analysis with the other *Streptococcus* genome to determine its unique dairy-specific properties. Genome-scale analysis of *S. thermophilus* MTCC 5460 revealed the presence of gene encoding for dairy-specific features including acid tolerance, lactose utilization, bacteriocin synthesis, production of formic

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and folic acids, rapid acidification of milk, bacteriophage resistance, exopolysaccharide (EPS) biosynthesis and genes involved in competence development.

## Materials and method

### Strain

The strain *S. thermophilus* MTCC 5460 (earlier named as *S. thermophilus* MD2), was an isolate from a market dahi (curd) sample collected by the Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India.

### Genome sequencing and assembly

The whole genome sequencing of *S. thermophilus* MTCC 5460 was performed using 454 GS-FLX (Roche) and ion torrent PGM platforms according to the manufacturer's instruction. The resultant sequencing reads were assembled using GS De Novo Assembler V.2.6. The ribosomal database project (RDP) classifier was used to classify this strain into higher order taxonomy and to confirm its homology. 16S rDNA comparison was performed by local BLAST of the assembled contigs with the 16S rDNA database downloaded from NCBI via ftp. Reads were further subjected to GS reference Mapper software V 2.3 for aligning it to the nearest organism reference sequence (*Streptococcus thermophilus* LMG 18311) and generating a consensus genome sequence. The circular genome map was generated using CGView Server ([http://stothard.afns.ualberta.ca/cgview\\_server/](http://stothard.afns.ualberta.ca/cgview_server/)).

### Gene prediction and annotation

The genome annotation of MTCC 5460 was performed with Rapid Annotation using Subsystem Technology V.4.0 (<http://rast.nmpdr.org/rast.cgi>) and Prokaryotic Genomes Automatic Annotation Pipeline (<http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>). The generated data output in the form of FASTA was uploaded in the RAST server for comparative analysis with the RAST job Id No. 1073569.6. The comparative functional analysis was carried out between the nearest strains of *S. thermophilus* publically available in the seed system of the RAST server.

### Genome inferred fermentative profile

The API 20 CHL test kit and API CHL medium (bio-Mérieux, La Balme les Grottes, France) were used to verify the abilities of *S. thermophilus* MTCC 5460 to ferment 20 carbohydrate moieties as per the manufacturer's instructions. Results of the biochemical analysis were validated with the presence of gene coding for the corresponding sugar metabolizing enzymes.

### Nucleotide accession number

The complete genome sequence of *S. thermophilus* MTCC 5460 can be accessed at Gene bank under accession no. ALIK00000000.

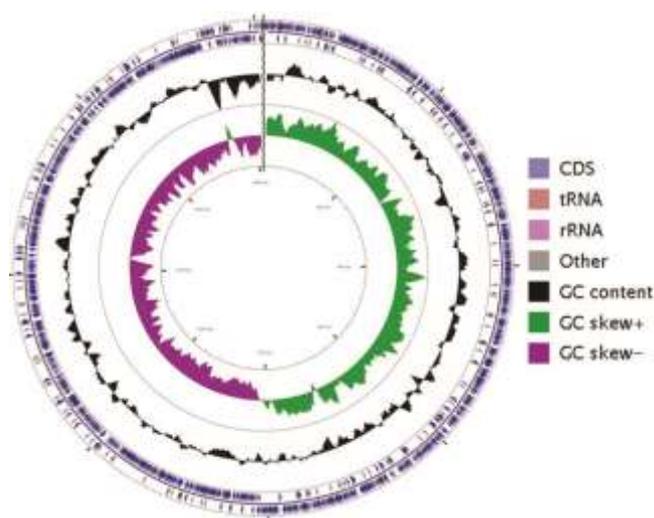
## Results and discussion

### Genome sequencing and assembly

Genome sequencing of *S. thermophilus* MTCC 5460 using NGS platform resulted in a total number of 37248425 bases generated from 307558 reads. The reference-based assembly resulted in an average contig size of 12626 bp. The N50 contig size was observed to be 23098 bp.

### General genome feature of *S. thermophilus* MTCC 5460

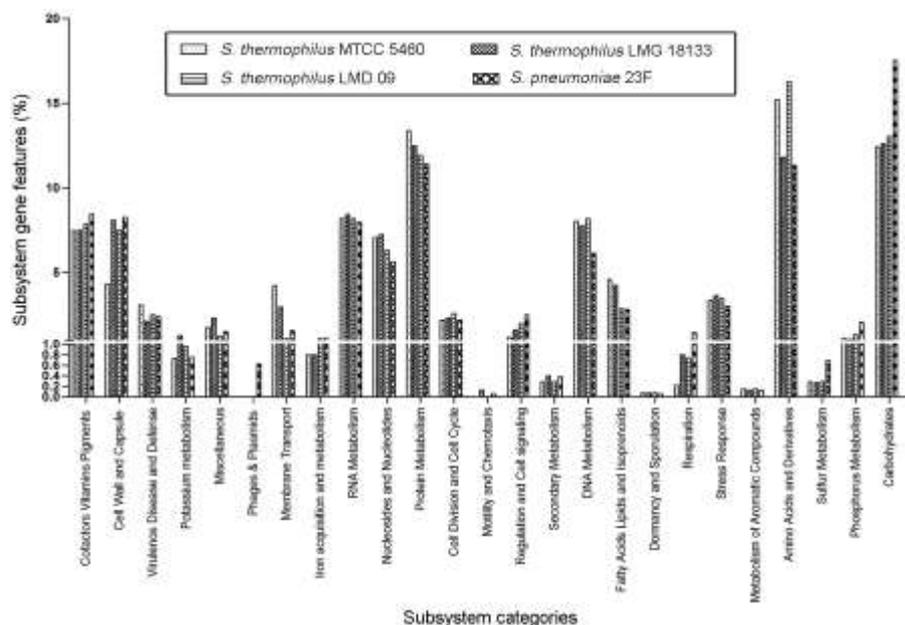
The plasmid free circular genome of *S. thermophilus* MTCC 5460 consisted of 16,09,365 bp. The GC content of *S. thermophilus* was 39.3%. The circular genome map of the isolate showing coding sequences, RNA genes, GC content and GC skew is shown in Figure 1. A comparison of general features of *S. thermophilus* MTCC 5460 and the three sequenced strains is shown in Table 1. Genome analysis of *S. thermophilus* MTCC 5460 revealed that the genome size and GC content are similar to other *Streptococci*. The ribosomal database project results confirmed the taxonomy by giving classification outcome as Domain: Bacteria; Phylum: Firmicutes; and Genus: *Streptococcus*. Local BLAST showed 98% identity with *S. thermophilus* ATCC 19258 strain.



**Figure 1.** Circular genome map of *S. thermophilus* MTCC 5460. The map represents the circular view of the genome sequence of *S. thermophilus* MTCC 5460. The circle was created using CG View server for online map creation. The outermost two rings depict coding sequences (CDS) and RNA coding regions on the (+) and (-) strands. Circle 3 shows the G + C content. Fourth and fifth rings show the GC Skew in the (+) and (-) strands respectively.

**Table 1.** Comparison of general genome features of studied strain MTCC 5460 with nearest publicly available *Streptococcus thermophilus* strain MTCC 5461, strains LMG 18311 and CNRZ 1066

Features	<i>S. thermophilus</i> MTCC 5460 Market dahi	MTCC 5461 Market dahi	LMG 18311 Yogurt isolate	CNRZ 1066 Yogurt isolate
Origin of strain	India	India	UK	France
Genome size (bp)	1,609,365	1,732,763	1,796,846	1,796,226
G + C content (%)	39.3	39.3	39	39
Coding sequences	1809	1764	1890	1915
No. of tRNAs	48	59	67	67
No. of Genes	1764	1766	1893	1903

**Figure 2.** Comparative functional annotation of *S. thermophilus* MTCC 5460 with other dairy strains and a pathogenic *Streptococcus* species.

### Gene prediction and annotation

Genome annotation by RAST revealed that the data set of MTCC 5460 contains 303 subsystems. A total of 1809 coding sequences (CDSs) and 58 structural RNAs were predicted. Out of 58 RNA genes, 48 were coded for tRNA and 10 for rRNA. Hypothetical proteins with unknown function were 13.2% of total CDS. The genome of *S. thermophilus* MTCC 5460 compared to that of LMG 18311 revealed that the number of genes under the categories of amino acid, membrane transport, virulence, disease and defence was higher in the former organism, indicating that the above functional groups may be associated with the adaptation of *S. thermophilus* MTCC 5460 to milk environment (Figure 2) and the gene coding for photosynthesis, motility and nitrogen metabolism was absent. The genome showed the presence of some *Eps* genes, homologous to *cps* and involved in synthesis of exopolysaccharide, which are important for industrial use of *S. thermophilus* as they confer the desired texture to yogurt<sup>11</sup>. The *cps* gene, responsible for synthesis of the capsule is vital for virulence in pathogenic streptococci

such as *S. pneumoniae*, whose absence in the genome confirms the non-capsulated feature of *S. thermophilus*. CRISPR/Cas system acting as a prokaryotic defence system against bacteriophage infections has been reported in the genome. MTCC 5460 possesses CRISPR/Cas system which might play an important role in the adaptive immunity, vital for dairy industries to safeguard against phage infection that causes failure of milk fermentations<sup>12,13</sup>. Earlier studies have also discussed the role of cas system in starter cultures and is also used as a parameter for comparative analysis of different *S. thermophilus* strains<sup>14</sup>. Other than these, the genome revealed the absence of many genes found in other streptococci. The organism MTCC 5460 did not reveal apparent antibiotic modification genes found in *S. agalactiae*. The absence of streptococcus virulence-related genes (VRGs), virulence determinants such as PspA, PspC, the pneumococcal manganese ABC transporter lipoprotein, pneumococcal choline-binding proteins, indicated that the strain MTCC 5460 is non-pathogenic. The presence of VRGs in *S. pyogenes* and the *S. pneumoniae* and absence in *S. thermophilus* indicate evolutionary divergence. Unlike its

pathogenic relative, absence of Rec Q indicates that it is not involved in the genome stability of MTCC 5460, similar to MTCC 5461 (ref. 15).

### Proteolytic system

The genome of MTCC 5460 revealed the presence of a variety of proteolytic system components such as cell wall associated proteinase like serine protease gene *htrA/degP* and foldase protease *prsA*. The MTCC 5460 genome does not possess a typical PrtP CEP system, but instead has shown housekeeping protease, *HtrA*, that is responsible for degradation of proteins, and is essential for growth at elevated temperatures<sup>16,17</sup>. *HtrA* showed 99% identity with *S. thermophilus* LMG18311 and ND03. A variety of cytoplasmic peptidases, metallopeptidases and transport system related to the efficient proteolytic metabolism were observed. The MTCC 5460 possesses cytoplasmic peptidases (*pepA*, *pepC*, *pepF*, *pepM*, *pepN*, *pepO*, *pepP*, *pepQ*, *pepT*, *pepV*, *pepX*) similar to those present in the genome of LMD-9, LMG 18311, CNZR 1066 and MTCC 5461 strains. This confirms its characteristic as a dairy starter. Oligopeptide transporters are important during growth in milk, for the uptake of peptides and amino acids<sup>18,19</sup>. This could be the probable reason behind the presence of OppABCDF peptide transport system in the strain genome. Lactocepin, a cell wall proteinase reported in *Lc. lactis* is known to impart bitterness in cheddar cheese. To overcome this defect the gene exchange is required<sup>20</sup>. The absence of lactocepin encoded gene in MTCC 5460 reveals its beneficial role as a dairy starter when compared to *Lc. lactis*. In addition to these, the genome possesses permease porters for the uptake of amino acid, polar amino acid transporter, branched chain amino acid transporters, one glutamine ABC transporter and one spermidine/putrescine ABC transporter permease as described in [Supplementary Table 1](#). The genome only lacks enzyme proline peptidase (*PepE/PepG*) and endopeptidase (*PepI/PepR/PepL*)<sup>21</sup>. Thus overall, a specific and defined proteolytic system was observed in MTCC 5460.

### Stress response

The genome of MTCC 5460 encodes a variety of stress proteins, viz. a proton translocating  $F_0-F_1$  ATPase system, urease system, ammonia permease and cation transport for acid resistance as shown in [Supplementary Table 2](#). These systems may contribute to maintaining an internal pH homeostasis. Cotter and Hill<sup>22</sup> suggested that *Lactobacilli* strains are categorized as probiotic when bacteria have the capacity to survive through shifting pH encountered in the gut. Remarkably, the urease system is only described in the *S. thermophilus* among all the species of LAB<sup>23</sup>. *S. thermophilus* can cope with oxidative

stress both in maintaining an appropriate intracellular redox environment and detoxifying ROS with antioxidant enzymes<sup>3</sup>. To deal with oxidative stress MTCC 5460 possesses superoxide dismutase, glutathione reductase, thioredoxin, thioredoxin dismutase, NADH oxidase and methionine sulphoxide reductase similar to their presence in the genome of ASCC 1275, LMD9 and *L. acidophilus* NCFM. In addition, several heat shock proteins (Hsp), cold shock proteins (Csp) and salt resistance genes were found in the genome. The heat shock related negative regulators *CtsR*, *HrcA* as well as chaperones *GrpE*, *DnaK*, *DnaJ*, *GroEL*, and *GroES* play a key role in bacterial responses to the sudden increase in environmental temperature by assisting protein folding. The probiotic organism carries *HrcA*, whereas the dairy strain lacks the repressor<sup>24</sup>. Like other probiotic bacteria, to cope with heat resistance, MTCC 5460 possesses *GroEL*–*GroES* system and *GrpE*, *DnaK*, *DnaJ* along with *HrcA*<sup>1</sup>. Glutamate production is reported to have an effect on acid production and acid tolerance in both Gram-negative and Gram-positive organisms. The MTCC 5460 reported the glutamate transporter involved in acid resistance<sup>25</sup>. Several salt resistance genes found in MTCC 5460, assist in cheese starter where salting is an essential step.

### Defence system

The MTCC 5460 genome encodes for histidine kinases and response regulators involved in two component regulatory system. Remarkably, MTCC 5460 genome possesses loci encoding for multidrug resistance, competence protein for natural transformation and bacteriocin production, details of which are listed in [Supplementary Table 3](#). The MTCC 5460 reported genes such as membrane-bound gene tRNA pseudouridine F synthase A, *dedE*, *purF* (amidophosphoribosyltransferase) engross in bacteriocin (*colicinV*) production similar to MTCC 5461 (ref. 15). Along with that, it possesses ABC-type bacteriocin exporters, contains an N-terminal double-glycine peptidase domain and is a cluster based system that involves eight genes. These genes are known to act as an alternative to food additives like potassium nitrate to prevent food spoilage by *Clostridia* as well as the growth of other foodborne pathogens<sup>26,27</sup>. Further, gene encoding for  $\beta$ -lactamase obtained via lateral gene transfer (LGT) during its evolution and multidrug resistance were found in the genome of MTCC 5460. In addition, the number of early and late competence genes was reported in MTCC 5460 genome. It has been demonstrated that the oligopeptide transporter, signal peptide and *comX* (sigma factor) that are important for induction of early competence development in *S. thermophilus*, were found in MTCC 5460. Regardless of its position as non-competent, the *S. thermophilus* genome appears to encode all late competence genes, which was also examined in several other bacterial genomes of non-competent bacteria such as *Lc. lactis*<sup>28</sup>.

### Biosynthetic capability

In the silico analysis of the genome, MTCC 5460 showed that it contained all the gene coding for the enzymes essential for biosynthesis of all amino acids with the exclusion of histidine. In addition, most cofactors and vitamin synthesis pathway related genes were found in the genome. Amino transferases and glutamate dehydrogenase were also prevalent in the genome of MTCC 5460. Apart from the above enzyme correlated with imparting flavour, several esterases encoding CDS were also present in the genome. Single-amino-acid omission analysis revealed that the number and the type of essential amino acids required for growth were strain-dependent<sup>29,30</sup>. Similar to other streptococci, silico analysis of MTCC 5460 revealed that the strain has the ability to synthesize three main precursor amino acids – aspartate, serine and cysteine from which series of other amino acids could be synthesized<sup>31</sup>. In *S. thermophilus*, it has been demonstrated that the activity of phosphoenolpyruvate carboxylase (Ppc) is essential for the bioavailability of L-aspartic acid during growth in milk. MTCC 5460 genome reported gene (IQ5\_03581) for this enzyme. Depletion of glutamate and glutamine or sulphur containing amino acids, methionine and cysteine stops the growth of *S. thermophilus*<sup>3</sup>. Fortunately, MTCC 5460 reported the capability to synthesize these amino acids. A complete *his* gene cluster region was deleted in CNRZ1066 and LMG18311 whereas it was present in the partial sequence of the LMD9 genome. Likewise, *his* gene cluster was not present in MTCC 5460. Like others, *S. thermophilus* MTCC 5460 was able to synthesize most of the co-factors and vitamins such as vitamin B6, riboflavin, nicotinate, nicotamide biotin, and folate with the exception of pantothenate CoA. The presence of aminotransferases AraT and BcaT along with the gene for GDH, corroborates its ability to degrade amino acids into aroma compounds. Similarly, these genes were also reported in other LAB used as dairy starters<sup>32</sup>. Amino acid catabolism adds flavour to the fermented dairy foods especially cheese and is also known to be strain-specific; hence this property can be further explored in MTCC 5460 which is used as a potential starter.

### Genome inferred fermentative profile

*S. thermophilus* MTCC 5460 was able to ferment a variety of sugar moiety. Sugar fermentation profile assessed with API 20 CHL demonstrated positive results for lactose, sucrose, fructose, trehalose, ribose, mannitol. Genome analysis of *S. thermophilus* revealed the presence of carbohydrate utilization genes which include four intact phosphotransferase systems specified for fructose, sucrose, mannose, maltose, and one truncated trehalose PTS system as shown in [Supplementary Table 4](#). In addition to these three intact sugar hydrolases, it includes a beta galactosidase, sucrose-6 phosphate hydrolase, cyto-

plasmic alpha amylase and lactose permease. The main role of *S. thermophilus* in dairy fermentations is not only in the rapid conversion of lactose into lactate but also the production of other compounds that contribute to flavour and texture<sup>3</sup>. Carbohydrate transport in bacteria is mediated via phosphotransferase systems (PTS) or ABC transporter. MTCC 5460 possesses PTS specific for fructose, sucrose, mannose similar to other *S. thermophilus* strains. Since lactose is the main sugar in milk, rapid acidification of milk by this starter is highly dependent on the utilization of lactose during milk fermentation<sup>33</sup>. The MTCC 5460 encode genes, to ferment lactose and convert it into short chain fatty acid apart from lactic acid and other substances. Probiotics can be developed as an effective therapy to prevent and diminish the symptoms of lactose intolerance. MTCC 5460 possesses pathways to convert N-acetylglucosamine to fructose, one of the essential substrate of glycolysis; this indicated that the organism is able to utilize N-acetylglucosamine as a carbon source apart from lactose. The MTCC 5460 possesses *lac-gal* gene cluster similar to that present in other sequenced streptococci. However, *L. bulgaricus*, a companion culture for *S. thermophilus* during yogurt fermentation, only has a *lacSZ* operon<sup>1</sup>. In addition to L-lactate, the main fermentation product, low levels of formate, acetoin and acetate have been found as additional end-products in MTCC 5460 (refs 34, 35). Further, genes involved in carbohydrate utilization regulation were ptsK HPrkinase/phosphorylase, ccpA-catabolite control protein, and ptsH-phosphor carrier protein HPr indicating an active carbon catabolite repression based on sugar availability<sup>31</sup>. Previous reports suggested that carbohydrate utilization capability promoted the ability to compete in specific environmental niche.

In conclusion, analysed genome sequence of *S. thermophilus* MTCC 5460 revealed that this bacteria is an extremely potent functional dairy starter reflected by the presence of systems for metabolizing lactose, the presence of amino acid and peptide, numerous stress response and host defense mechanisms. Remarkably, its well defined proteolytic system may attribute the adaptation of this organism to milk environment. Plenty of stress response and multidrug resistance genes allow MTCC 5460 as a probiotic candidate. Nevertheless, sequence data information provides details about the absence of avirulent gene, making it a non-pathogenic organism. Further, it exhibited genes for numerous characteristics, including that for safety, appearance, as well as flavour and aroma that formulate it as a functional dairy starter culture as well as a promising potential probiotic.

**Conflict of interest:** The authors declare that they have no conflict of interest.

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

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