

Molecular characterization and functional analysis of the manganese-containing superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* AO54[☆]

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Abstract

This report describes the isolation, sequencing, and functional analysis of the *sodA* gene, encoding Mn-superoxide dismutase, from *Streptococcus thermophilus* AO54. The gene was found to encode a 201 amino acid polypeptide with 88 and 83% identity to SodA from *Streptococcus mutans* and *Streptococcus agalacticae*, respectively. Primer extension analysis revealed a transcriptional start site 27 nucleotides upstream of initiation codon. The gene was expressed in *Escherichia coli* and was able to rescue the growth of a *sodAsodB* mutant in a minimal-medium containing 10^{-6} M paraquat. A *sodA* mutant of *S. thermophilus* was constructed and found to be more sensitive to aerobic growth than its parent strain. Supplementing the medium with $MnCl_2$ improved the growth of the mutant, only under microaerophilic conditions. The results suggest that *sodA* is essential for the aerobic growth of *S. thermophilus*. In the absence of functional SodA, manganese ions may provide partial protection against oxygen toxicity.

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Keywords: *Streptococcus thermophilus*; Superoxide dismutase; MnSOD; *sodA* sequence; *sodA* mutant; SOD-null mutant; Oxygen sensitivity; Manganese ions; DNA polymerase III

Lactic acid bacteria (LAB)¹ are Gram-positive, non-sporulating, acid-tolerant organisms capable of producing lactic acid via fermentation [1]. Generally, they are regarded as aerotolerant anaerobes [1] and many possess oxygen-utilizing enzymes, such as pyruvate oxidase [2–6] and NADH oxidase [7–11]. Consequently, partially reduced, highly reactive oxygen intermediates may be generated during metabolism in the presence of air. These reactive oxygen species (ROS) include the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2),

and the hydroxyl radical (HO^{\cdot}). In general, ROS have been demonstrated to be both cytotoxic [12] and mutagenic [13,14]. Organisms have evolved antioxidant defense systems to cope with oxidative stress. These systems include the antioxidant enzymes superoxide dismutases (SODs) and hydroperoxidases (HPs), which detoxify superoxide radical and hydrogen peroxide, respectively, and thus prevent the formation of hydroxyl radical [12].

Superoxide dismutases (EC 1.15.1.1) are metalloenzymes that catalyze the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide [15]. There are three isoforms of SODs that are distinguished by the type of metal found at their catalytic center: manganese, iron, and copper [12]. Organisms may employ one, two or all three isoforms of SOD for cellular protection against oxidative stress. *Escherichia coli*, for example, contains all three isoforms [16–18]. Likewise, many LAB

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¹ Abbreviations used: LAB, lactic acid bacteria; ROS, reactive oxygen species; SODs, superoxide dismutases; HPs, hydroperoxidases; orfs, open reading frames; Cm, chloramphenicol; Km, kanamycin; Tet, tetracycline; Erm, erythromycin.

have evolved such systems; superoxide dismutases have been identified in *Lactococcus lactis* [19] and in many species of streptococci [20–23]. Other strains of LAB that lack antioxidant enzymes may employ other free radical scavenging systems. For example, *Lactobacillus plantarum* offsets the lack of SODs by accumulating high intracellular concentrations of manganese ions, which are capable of scavenging superoxide radical [20,24].

Streptococcus thermophilus is a homofermentive, thermophilic streptococci, and is known to contain an NADH oxidase [25]. Previous work in our laboratory has shown that *S. thermophilus* possesses only one type of SOD, the Mn-containing enzyme (MnSOD) [21]. The activity of MnSOD was found to increase in a growth-dependant fashion, increasing three- to fourfold upon entry into stationary phase [21]. Furthermore, the activity of the enzyme was not increased in response to the presence of the redox-cycling compound paraquat, nor was there differential expression of the enzyme under aerobic vs. anaerobic condition [21].

In this study, we report the isolation and the sequence of the *sodA* gene, encoding for the MnSOD, from *S. thermophilus*. The gene was disrupted and the physiological role of the gene product SodA (MnSOD) was investigated.

Materials and methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* were grown either at 37 or

30 °C in Luria–Bertani (LB) medium or LB supplemented with 0.5% glucose (for anaerobically grown cultures) and supplemented with the appropriate antibiotics when required. For *E. coli* LE392, used for transduction, LB was supplemented with 0.2% maltose and 10 mM magnesium sulfate. When required, the antibiotics used with *E. coli* cultures were at the following concentrations: chloramphenicol (Cm, 20 µg/ml), kanamycin (Km, 50 µg/ml), tetracycline (Tet, 20 µg/ml), and erythromycin (Erm, 200 µg/ml). *E. coli* MV12 was grown at 37 °C in Mueller–Hinton broth supplemented with 100 µg/ml trimethoprim. For selection of *sodA* transformants, transformed NC906 cells were plated onto M-9 minimal medium plates [26] containing 0.5% glucose, the appropriate antibiotics, and 10⁻⁶ M paraquat [27]. *S. thermophilus* AO54 was grown at 42 °C in M17 media supplemented with 0.5% glucose (M17G). When required, the antibiotic erythromycin was added to the *S. thermophilus* cultures at a concentration of 2 µg/ml. For anaerobic growth, cells were grown in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). For preparing electrocompetent *S. thermophilus*, cells were grown in Belliker broth [28] (Elliker broth [29] supplemented with 1% beef extract). Plates of all the aforementioned media were made by supplementing the appropriate media with 1.2% agar.

Materials and enzymes

Paraquat, cytochrome *c*³⁺, xanthine, xanthine oxidase, riboflavin, nitroblue tetrazolium, lysozyme, proteinase K, phenol, chloroform, and all antibiotics used in this study were purchased from Sigma (St. Louis,

Table 1
Strains, vectors, and phages

Strains	Relevant characteristics	Source
<i>Escherichia coli</i>		
DH5α	<i>rec</i> ⁻ cloning strain	Stratagene
LE392	<i>rec</i> ⁺ , lambda permissive	Promega
NC906	<i>ΔsodA</i> , <i>sodB</i> , Km ^r	[31]
PCN-8	as NC906 but contains pLAFR-2 + <i>S. thermophilus</i> DNA fragment	This study
PCN-9	as NC906 but contains pLAFR-2 + <i>S. thermophilus</i> DNA fragment	This study
DH-SodA	as DH5α but contains pSODA-4.4	This study
MV12	provides genetic transfer functions via pRK2073	S. Libby
MCK12	<i>rec</i> ⁺	Stratagene
<i>Streptococcus thermophilus</i>		
AO54	wild type industrial strain	[32]
KO 2-4	AO54 <i>ΔsodA</i>	This study
Phage		
Package lambda phage		Promega
Vectors		
pLAFR-2	Tet ^r cloning cosmid	S. Libby
pBluescript SK+	amp ^r cloning vector	Stratagene
pRK2073	contains mobilization genes for transfer	S. Libby
pG ⁺ host9	erm ^R temperature-sensitive Integration shuttle vector <i>ΔISS1</i>	[34], T. Klaenhammer
pKO-1	Same as pG ⁺ host9, but with 500 bp <i>sodA</i> PCR fragment	This study

Mo). All other chemicals were purchased from Fisher Scientific (Pittsburgh, Pa). Bacteriological media from Difco were purchased from Fisher Scientific (Pittsburgh, Pa). Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, Klenow fragment, and reverse transcriptase were purchased from Promega (Madison, WI), New England BioLabs (Beverly, Mass.), Qiagen (Valencia, CA), or Roche (Indianapolis, IN). All radiochemicals were purchased from ICN (Irvine, CA).

DNA isolation and manipulation

Isolation of plasmids from *E. coli* was performed by using the Qiagen Mini Spin isolation kit (Qiagen, Valencia, CA) according to supplier's instructions. Isolation of cosmid DNA was carried out using the Qiagen Midi Spin (Qiagen) isolation kit according to supplier's instruction. Chromosomal DNA isolation was carried out as follows: ten milliliters of overnight cultures of *S. thermophilus* was pelleted by centrifugation at 8000g, 4 °C for 10 min. The cells were resuspended in 2.5 ml of lysis solution (6.7% sucrose, 50 mM Tris-HCl, and 1 mM EDTA, pH 8.0), to which 1 ml of 50 mg/ml lysozyme was added, and the suspension was allowed to incubate at 37 °C for 1 h. Following the incubation, SDS was added to a final concentration of 1% and the suspension was incubated at 60 °C until clear. Twenty milliliters of proteinase K (20 µg/ml) was added. The suspension was allowed to incubate at 60 °C for another 20 min. The suspension was extracted twice with phenol:chloroform and the aqueous phase were transferred to a tube containing ethanol. The resulting DNA was spooled onto a sterile glass Pasteur pipette and dissolved in 250 µl TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as per the recommendations of the respective suppliers. DNA fragments were resolved by electrophoresis on 1% TAE agarose gels as described by Sambrook et al. [26]. DNA isolation from agarose gels was carried out using either Bio101 GeneCleanII (Bio101, La Jolla, CA) or a Qiagen Qia-Quick (Qiagen, Valencia, CA) isolation kit. Sequencing was performed at the Iowa State University Sequencing Facility. All sequence analysis was performed using BioEdit biological sequence alignment editor [30].

Construction and screening of genomic library

S. thermophilus genomic DNA was extracted and digested for different lengths of time with *Mbo*I. The partial digests were then analyzed on an agarose gel to determine the ideal digestion time to obtain DNA fragments about 20 kb in length. The 20 kb genomic fragments were gel purified, combined with *Bam*HI restricted pLAFR-2 vector along with T4 DNA ligase, and allowed to ligate at room temperature overnight.

The mixture was then added to the Packagene Extract (Promega, Madison, WI) of lambda phage particles. This mixture was incubated at room temperature for three hours and then diluted 1:1000 with phage buffer (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 10 mM MgSO₄). One hundred microliters of the phage was added to 10 µl *E. coli* LE392 cells grown to stationary phase with the appropriate supplements. The phage was allowed to adsorb at 37 °C for 45 min, fresh LB (1 ml) was added, and the cells was grown at 37 °C for 1 h to allow expression of the tetracycline resistance gene. Two hundred microliters of cells were then plated on LB-tetracycline (20 µg/ml) plates and incubated overnight at 37 °C.

Triparental mating

A triparental mating was performed to mobilize the cosmid library from the *E. coli* LE392 host into a *sodA sodB* mutant strain of *E. coli*, NC906 [31]. Equal volumes of log phase cultures of *E. coli* MV12 containing the pRK2115 plasmid, *E. coli* LE392 containing the cosmid library, and *E. coli* NC906 were mixed and concentrated by filtration through Swinex filters. The filters were placed onto LB plates and incubated for 5 h at 37 °C. The cells were then eluted with a saline buffer and plated onto M-9 medium selecting for the presence of the cosmid (Tet), the SOD deficient strain (Km), and the presence of a functional SOD (10⁻⁶ M paraquat).

Southern blot hybridization and DNA probes

DNA probes were made by random hexamer nucleotide labeling according to Ausubel et al. [33]. Unincorporated nucleotides were removed by using a ProbeQuant G-50 Micro Column (Amersham-Pharmacia Biotech, Piscataway, NJ). Southern blot analysis was carried out using radiolabeled probes as described by Sambrook et al. [26].

PCR

Standard polymerase chain reaction was carried out using Qiagen *Taq* DNA Polymerase (Qiagen, Valencia, CA) as per manufacturer's instruction. Primers STSODF (5'-GAGAGGACAGATTCAAGATG-3') and STSODR (5'-GTTTTGGCGGCTCC-3') (Integrated DNA Technologies, Coralville, Iowa) were used to amplify an approximately 1.2 kb DNA fragment containing the structural gene of *sodA* from *S. thermophilus* and flanking sequences, in particular the upstream region of the gene to include any promoter elements. Primers KOF (5'-GGAATTCCTTCCTTACGCTTACGATGTTTGG-3') and KOR (5'-GGAATTCCTCAGCAACTTTATTC-3') were purchased from Integrated DNA Technologies (Coralville, Iowa).

The 435 bp probe was synthesized using primers based on those previously reported by Poyart et al. [22]: PThermSod1 (5'-GACTCTTCATCATGAG-3') and PThermSod2 (5'-CCGTCTAGATAGGTG-3'), both purchased from Sigma. Primer PERM (5'-GGGTTGCTCTTGCACTC-3') used to verify insertional inactivation was purchased from Integrated DNA Technologies (Coralville, Iowa). All PCRs were carried out using a Perkin-Elmer Biosystems GeneAmp 2400 PCR System (Boston, Mass.).

RNA isolation and primer extension

Total RNA from *S. thermophilus* was isolated using the MasterPure RNA Purification Kit (Epicentre Technologies, Madison, WI) as per manufacturer's recommendations with the following modification. Cells were harvested by centrifugation at 5000g for 5 min at 4 °C and the pellet was resuspended in 100 µl of 3 mg/ml lysozyme in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated at room temperature for 15 min prior to using the MasterPure RNA Purification Kit (Epicentre, WI). Primer extension was carried out by mixing 20 µg total RNA with 1–5 pmol ³²P end-labeled primer, as described by Ausubel et al. [33]. The primer used for both the primer extension and sequencing, PX1 (5'-CAGCATCAATGTATGGTTCC-3'), was purchased from Integrated DNA Technologies (Coralville, Iowa).

Bacterial transformations

Escherichia coli were transformed either by CaCl₂ transformation method as described by Sambrook et al. [26] or by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) according to manufacturer's protocol. *S. thermophilus* transformation was carried out according to the protocol by O' Sullivan and Fitzgerald [28]. Transformants were selected in M17G broth containing erythromycin and grown to mid-log phase at 30 °C to allow the plasmid to propagate in the cells.

Insertional mutagenesis

For insertion mutagenesis experiments, the pG⁺host9 [34] was used to disrupt the *sodA* gene in *S. thermophilus* AO54. Briefly, a 500 bp internal *sodA* fragment was amplified using the primers KOF and KOR and *S. thermophilus* AO54 genomic DNA as a template. The fragment was cloned into the pGEMT-Easy PCR cloning vector (Promega) and then subsequently removed by digestion with *EcoRI*. The resulting 500 bp fragment was purified and cloned into the *EcoRI* restriction site in the pG⁺host9 plasmid, resulting in the new construct, a 4.3 kb plasmid, pKO-1. The pKO-1

plasmid was transformed into *S. thermophilus* AO54 via electroporation [28]. Transformants were used to inoculate 100 ml M17G broth supplemented with erythromycin and allowed to grow at the permissive temperature, 30 °C, under anaerobic conditions. One hundred microliters of cells from the culture was spread onto fresh M17G plates supplemented with erythromycin and allowed to grow at the non-permissive temperature, 42 °C [34], under anaerobic condition. Putative clones were screened for the appropriate marker(s) and insertion of the plasmid was confirmed by PCR.

Preparation of cell-free extracts

Cells were harvested by centrifugation at 5000g for 30 min at 4 °C. The cells were washed in 0.05 M phosphate buffer containing 10⁻⁴ M EDTA (pH 7.8) (KPi-EDTA buffer), pelleted by centrifugation at 8000g, 4 °C for 10 min, and resuspended in the same buffer. The cell suspensions were then disrupted by bead beating with a MiniBeadbeater-8 (Biospec Products, Bartlesville, OK) for 6 × 1-min intervals, a total of 6 min. Overheating was prevented by placing the tubes on ice for three minutes between treatments. Cellular debris was removed by centrifugation at 14,000g, 4 °C for 30 min. The supernatant was dialyzed at 4 °C, for 24 h, against three changes of the Pi-EDTA buffer.

Biochemical assays

Dialyzed cell-free extracts were assayed for protein concentration [35], using bovine serum albumin as standard. SOD activity in both *E. coli* and *S. thermophilus* was analyzed by activity gels using nitroblue tetrazolium [36] and in liquid using the cytochrome *c*³⁺ and xanthine/xanthine oxidase method [15].

Growth with MnCl₂

Overnight culture of *S. thermophilus* grown anaerobically at 42 °C in M17G broth was used to inoculate fresh anaerobic M17G broth with and without MnCl₂ (made anaerobic by placing in a Coy anaerobic chamber overnight) to an OD₆₀₀ of approximately 0.1. The two cultures (i.e., +/- MnCl₂) were allowed to grow anaerobically at 42 °C to an OD₆₀₀ of approximately 0.25 before each was divided into three cultures and incubated either in the Coy Chamber (anaerobic), in air without shaking (microaerophilic), or in air with shaking at 200 rpm (aerobic). Changes in the optical density of the six cultures were monitored at 600 nm (OD₆₀₀) and plotted against time (linear plots were used to allow for the identification of the OD₆₀₀ values).

Results

Isolation of *sodA* from *S. thermophilus*

A cosmid DNA library of *S. thermophilus* AO54 was mobilized into the *sodAsodB* mutant of *E. coli* (NC906) and screened for the ability to complement the lack of SOD by rescuing the growth of the mutant on M9 minimal salt media containing 10^{-6} M paraquat. Twelve putative clones were obtained from this screen. All clones examined contained the *sodA* gene and demonstrated superoxide dismutase activity. Fig. 1A shows SOD activity in two of the twelve clones. One of the clones, PCN-9, was selected for further study. The cosmid containing the *sodA* gene, PCN-9, was digested with *Bam*HI (Fig. 1B) and probed with a radiolabeled PCR-generated 435-bp internal fragment [22] of *sodA* from *S. thermophilus* (Fig. 1C). Fig. 1C shows a 4.4 kb fragment that hybridized to the *sodA* probe. The fragment was extracted and ligated into *Bam*HI-digested pBluescript SK⁺, and transformed into the *E. coli* DH5 α . A clone harboring the plasmid that contained the 4.4 kb insert that hybridized to the *sodA* probe was identified and designated as DH-SodA, and the plasmid was designated as pSODA-4.4.

Expression of *S. thermophilus sodA* in the *E. coli sodAsodB* Mutant

The pSODA-4.4 was functionally expressed in the *sodAsodB* mutant of *E. coli* (NC906), as evident of its ability to rescue the aerobic growth of the mutant on solid M9 medium containing 10^{-6} M paraquat. Furthermore, Table 2 shows the specific activity of MnSOD in the *E. coli* NC906 harboring pSODA-4.4 grown

Table 2

Expression of pSODA-4.4 in *E. coli* NC906

Growth condition	Specific activity (U/mg protein)
Aerobic with shaking (200 rpm)	11.9 \pm 0.50
Aerobic still	4.59 \pm 0.45
Anaerobic	3.68 \pm 0.62

E. coli NC906 was grown as in Materials and methods.

Dialyzed cell-free extracts were used to assay protein and SOD.

• Results are based on the average of three independent experiments.

under different growth conditions (i.e., anaerobic, aerobic still, and aerobic with shaking at 200 rpm).

Sequence analysis

The 4.4 kb fragment was sequenced (GenBank Accession No. AF538722). The sequence contained two open reading frames (orfs). Orf 1 was located upstream of orf 2 (ending 148 nucleotides upstream of the start codon of orf 2) and encodes a predicted protein of 344 amino acids. A BLAST search of the translated predicted sequence of orf 1 found it to be 84% homologous (69% identical) to a hypothetical protein in *Streptococcus agalactiae* [37] and *S. mutans* [38]. This hypothetical protein also exhibited 81% homology (65% identity) to the delta subunit of DNA polymerase III in *Streptococcus pyogenes* [39].

The nucleotide sequence of orf 2 contained the complete sequence of *sodA*. Comparison of this nucleotide sequence to a previously published partial sequence of *sodA* from *S. thermophilus* CIP 102303 [22] showed 99% identity over the 435 bp reported. However, two base differences were found at nucleotides #204 and #303 of

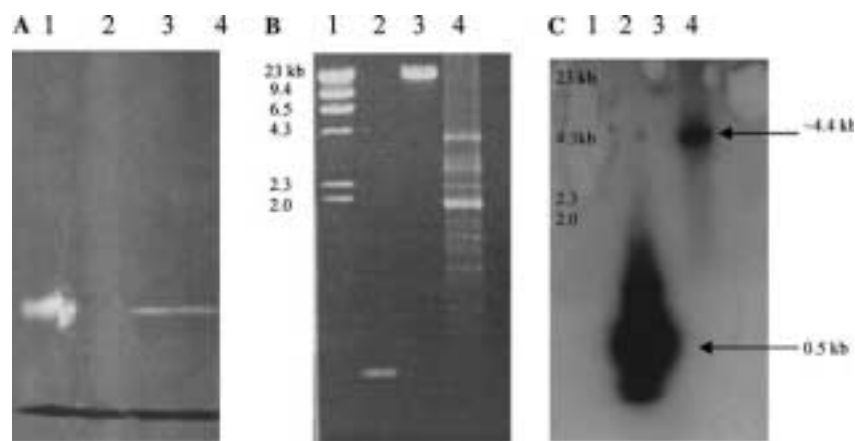


Fig. 1. Identification of MnSOD and *sodA* Gene from *S. thermophilus*. (A) Non-denaturing polyacrylamide protein gel stained for SOD activity. Fifty micrograms of cell-free extracts was loaded per lane. Lanes: 1, *S. thermophilus* AO54; 2, *E. coli* NC906; 3, *E. coli* NC906 with pCN-8; 4, *E. coli* NC906 with pCN-9. (B) 1% agarose gel showing *Bam*HI digest of pCN-9. Lanes: 1, 1 kb ladder; 2, 435 bp internal *sodA* fragment (probe); 3, pLAFR-2 cosmid vector; and 4, pCN-9 digested with *Bam*HI. (C) Southern blot hybridization of gel in panel B using a radiolabeled 435 bp internal *sodA* fragment. Lanes: 1, 1 kb ladder; 2, 435 bp internal *sodA* fragment (probe); 3, pLAFR-2 cosmid vector; and 4, pCN-9 digested with *Bam*HI. Note. The gel and the radiograph are not to scale.

the published partial sequence [22], where c is changed to t, and t to c; respectively. These changes, however, were silent with regard to the predicted amino acid sequence. While a conserved streptococcal ribosomal binding site was present, no consensus –35 and –10 regions were apparent. Orf 2 was found to encode a 201 amino acid protein that was homologous to the manganese-containing superoxide dismutase found in many bacterial

species. The predicted amino acid sequence was 88% identical to SodA from *S. mutans* [40], 83% identical to *S. agalactiae* [37], 62% identical to *Lactococcus lactis* [19], 57% identical to *Staphylococcus aureus* [41], 61% identical to *Bacillus subtilis* [42], and 50% identical to *E. coli* [43], and *Salmonella typhimurium* [44]. Fig. 2 shows the predicted amino acid sequence alignment for these SodA sequences.

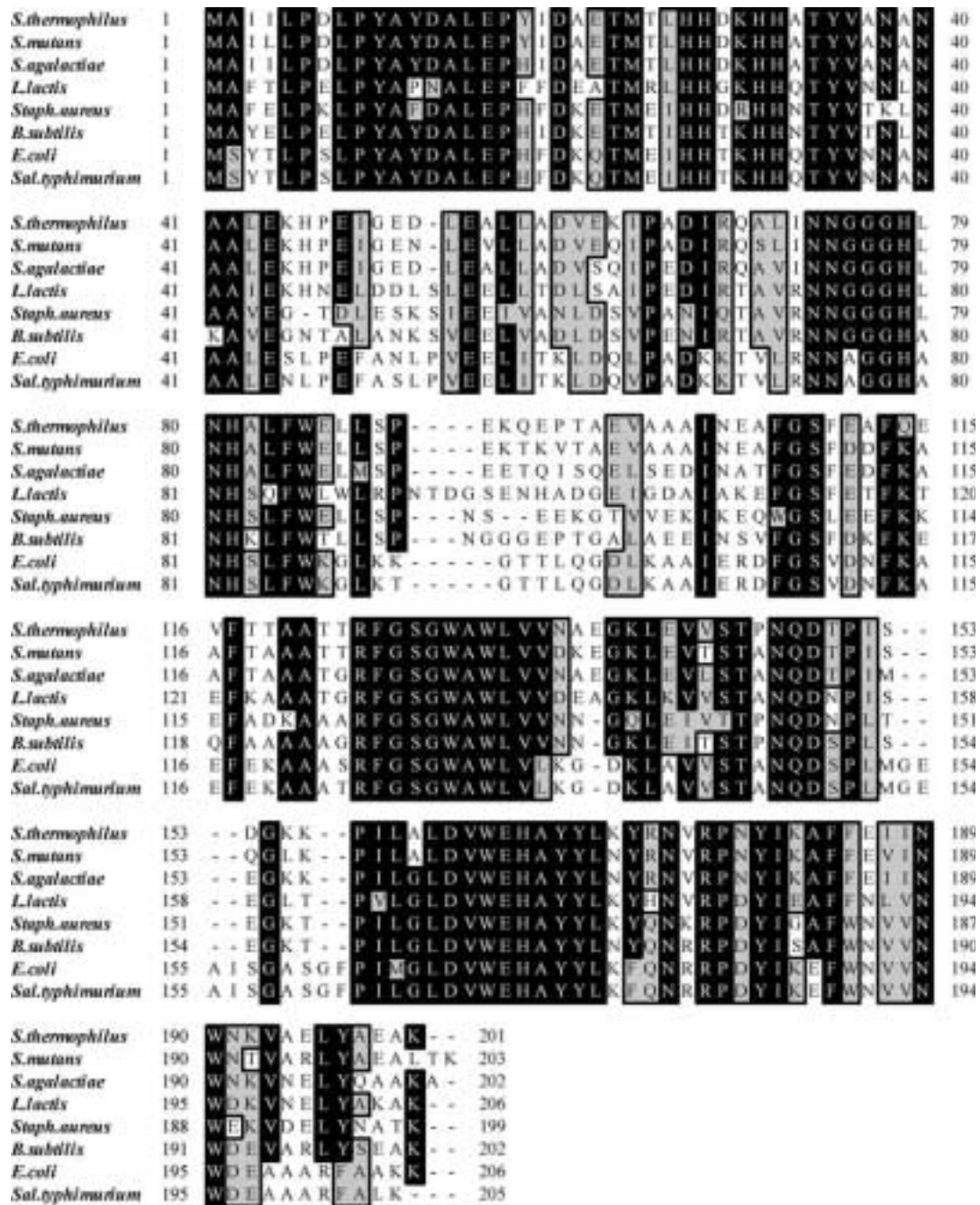


Fig. 2. Alignment of the predicted amino acid sequence of *S. thermophilus* sodA with other SodA sequences. Alignment was performed using Clustal × sequence alignment program. Black shading represents amino acids that are identical, gray shading represents amino acids with at least 70% homology.

Primer extension analysis

RNA from anaerobically grown *S. thermophilus* cells entering stationary phase was extracted and used as a template for primer extension. The primer PX1, located 44 nucleotides downstream from the translational start site, was used to ascertain the beginning of the mRNA encoding *sodA*. The data indicated (Fig. 3) that the transcriptional start site is at the G located 27 base pairs upstream of the translation start site.

Physiological function of *SodA* in *S. thermophilus* AO54

To investigate the physiological role of MnSOD (*SodA*) in *S. thermophilus*, a *sodA* mutant of *S. thermophilus* was constructed, using the Gram-positive temperature sensitive integration vector pG⁺host9 [34]. A schematic diagram of the insertional mutagenesis strategy is illustrated in Fig. 4A and B. The putative (*erm*^R) mutants were selected under anaerobic conditions due to their potential sensitivity to oxygen. Insertion of the 4.3 kb pKO-1 plasmid was tentatively verified

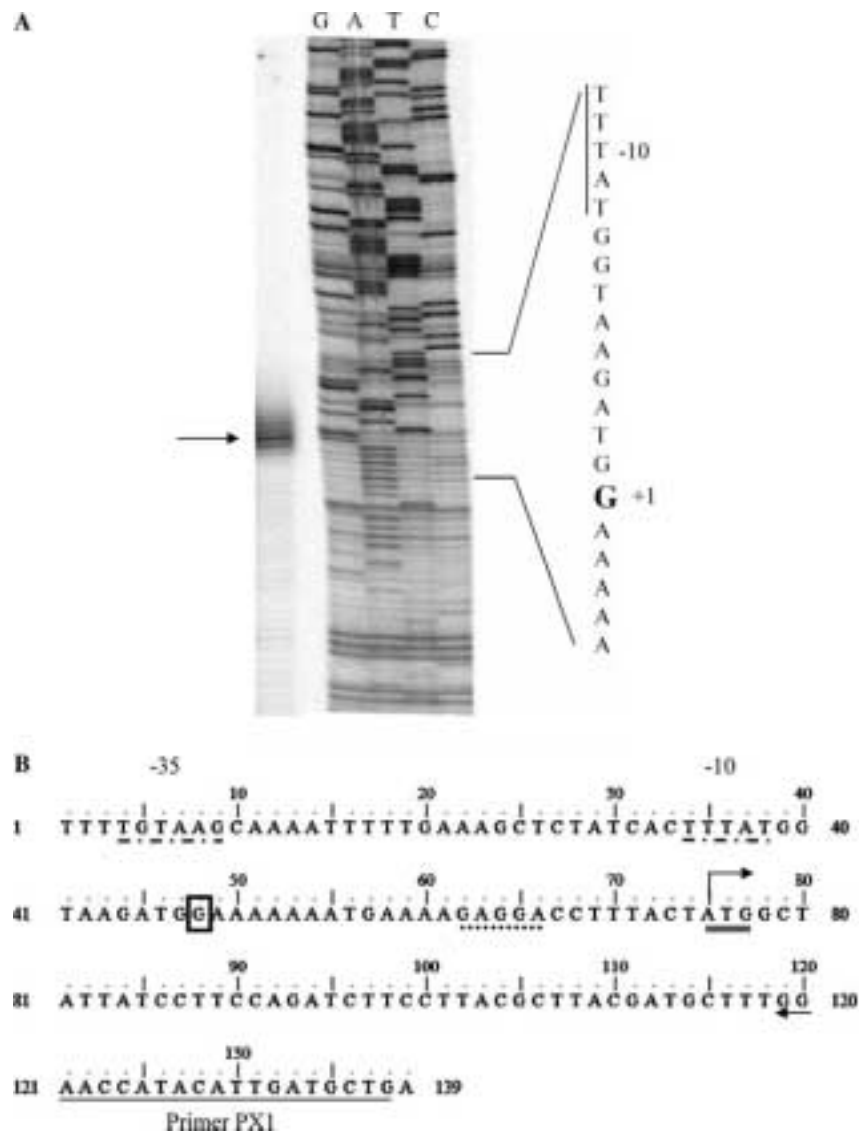


Fig. 3. Primer extension analysis of the 5' end of the RNA transcript of the *sodA* gene from *S. thermophilus*. (A) The transcriptional start site was mapped 27 nucleotides upstream of the ATG start codon using the primer PX1. The sequence written to the right of the sequencing gel shows the guanine (bold capital G, +1) that begins the RNA transcript, as well as the -10 site. The lane to the left of the sequencing lanes contains 5 μ l of the primer extension reaction. The arrow indicates the major primer extension product. (B) Sequence below showing PX1 primer location relative to the coding sequence, ATG start codon (double underlined sequence with arrow indicating direction of transcription), predicted *S. thermophilus* ribosomal binding site dotted underlined sequence), start nucleotide (boxed letter), and putative -10 and -35 sequences (dashed underlined).

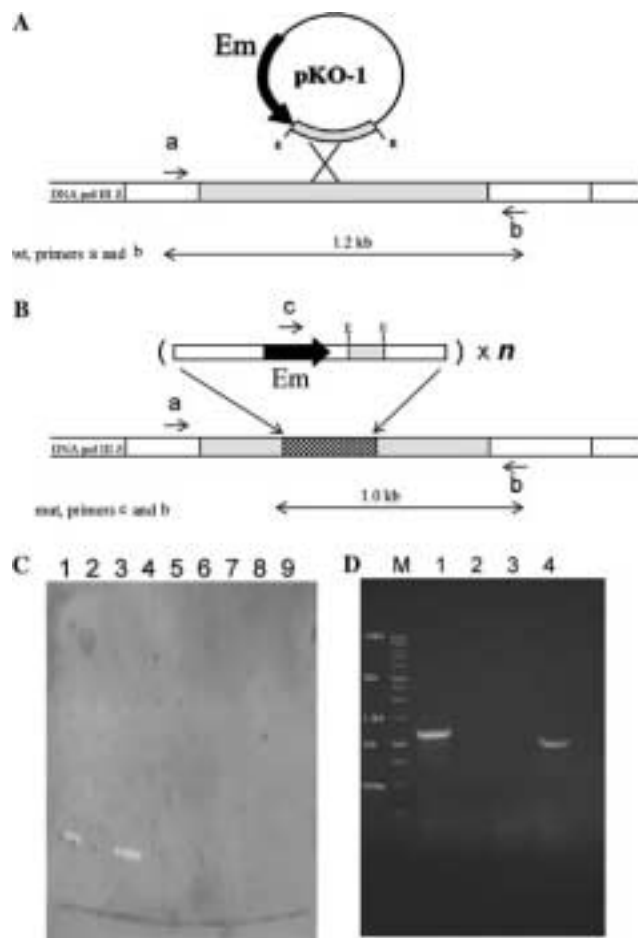


Fig. 4. Construction of a *S. thermophilus sodA* mutant strain. A 500 base pair internal fragment of *sodA* was PCR amplified with primers KOR and KOF containing the restriction site for *EcoRI*. The fragment was amplified, digested, and ligated into the temperature-sensitive integration vector pG⁺host9 [34]. The newly constructed vector, pKO-1 (4.3 kb), was transformed into the *E. coli* cloning strain MC1061. pKO-1 was subsequently transformed into *S. thermophilus* AO54 and grown at 30 °C under anaerobic conditions. A single cross-over event occurring within the genomic *sodA* sequence (A) results in an integration of the pKO-1 plasmid into the gene, thereby disrupting it (B). Primers STSODF (a), STSODR (b), PERM (c), and predicted PCR products are shown. Multiple copies of the integrated plasmid are possible (34) (designated with by "x n"). Transformants were recovered, plated, and grown at 42 °C under anaerobic conditions. Survivors (erm^R) were examined for plasmid and integrants were confirmed by PCR over the integration junctions (see text). Note. diagram not drawn to scale. (C) Non-denaturing polyacrylamide protein gel stained for SOD activity. Fifty micrograms of cell-free extract from nine putative disrupted clones of *S. thermophilus* AO54 was loaded per lane. Clear bands represent area of SOD activity. Lanes: 1, *S. thermophilus* AO54; 2, KO 1-1; 3, KO 1-2; 4, KO 1-3; 5, KO 1-4; 6, KO 2-1; 7, KO 2-2; 8, KO 2-3; and 9, KO 2-4. (D) A 1% TAE agarose gel of PCR products recovered from pKO-1 integrants in *S. thermophilus* AO54 genome by PCR. Lane M, molecular weight markers; Lane 1, AO54 with *sodA* primers a and b; Lane 2, AO54-KO2-4 with primers a and b; Lane 3, AO54 with primers c and b; and Lane 4, AO54-KO2-4 with primers b and c. Note. Figure is not drawn to scale.

by loss of SodA activity. Fig. 4C shows the lack of MnSOD in seven out of eight putative integrants. One of these clones, *S. thermophilus* KO2-4, was selected for

further investigation. PCR was used for the verification of the insertion, where a primer specific for an internal portion of the erythromycin resistance gene from the pKO-1 plasmid (PERM; designated as c) and a primer designed outside the chromosomal *sodA* sequence (STSODR; designated as b) were used (Fig. 4B). Using these primers would result in a 1 kb amplified fragment if the pKO-1 plasmid had been successfully integrated into the *S. thermophilus* chromosomal *sodA* locus. Fig. 4D depicts a 1% TAE agarose gel containing the products of PCRs where the genomic DNA from the parent AO54 and integrant KO2-4 were used as templates to verify the insertion of pKO-1. Fig. 4D (Lanes 1 and 2) shows the results of PCRs using the *sodA* specific primers (i.e., primers a and b). The predicted 1.2 kb product was amplified from the wild type AO54 DNA (Fig. 4D, Lane 1), but no product was recovered from the KO2-4 DNA (Fig. 4D, Lane 2). The lack of a PCR product from the reaction using the mutant DNA as a template (Lane 2) is most likely due to concatamerization of the integrant within the *sodA* sequence resulting in a template too large ($\gg 5.5$ kb) to amplify via conventional PCR methods. Fig. 4D (Lanes 3 and 4) shows the results of PCR reactions using a primer from within the *erm* gene (primer c) and a primer flanking the *sodA* sequence (primer b). No product was amplified when the wild-type DNA was used as template, thus indicating the absence of pKO-1 in the *sodA* locus (Fig. 4D, lane 3). A ~1 kb fragment was amplified from the integrant KO2-4 (Fig. 4D, lane 4). This was the expected size of the fragment using the PERM (c) primer (located approximately 300 bp away from the *EcoRI* restriction site in the pG⁺host9 plasmid) and the STSODR (b) primer, located approximately 200 bp away from the KOR primer. These results in addition to the loss of SodA activity supported the conclusion that *S. thermophilus* strain KO2-4 is a null-mutant of *sodA*.

To determine the sensitivity of the *sodA* mutant of *S. thermophilus* (KO2-4) to aerobiosis, cells were grown anaerobically at 42 °C on M17G media (containing erythromycin), harvested, serially diluted, and plated

Table 3
Sensitivity of *S. thermophilus* KO 2-4 to aerobic growth conditions

Strain	CFU/ml		Ratio
	Anaerobic	Aerobic	Aerobic: Anaerobic
<i>S. thermophilus</i> AO54	4.20E+07	6.50E+07	1.55
<i>S. thermophilus</i> KO2-4	1.60E+08	4.40E+04	2.75E-04

S. thermophilus AO54 and KO2-4 were grown anaerobically for 24 h in a Coy anaerobic chamber. Appropriate dilutions were made anaerobically and plated on M17G ± air.

* Results are based on the average of three independent experiments.

anaerobically onto two sets of fresh M17G plates containing erythromycin. One set of plates was kept in the anaerobic chamber and incubated at 42 °C, while the second set of plates was removed from the anaerobic chamber and incubated aerobically at 42 °C. The results in Table 3 show that the KO2-4 strain was markedly sensitive to aerobic conditions, resulting in a greater than 4-log reduction in the number of colonies formed under aerobic conditions as compared to those formed under anaerobic conditions. The few colonies of KO2-4 that appeared on the aerobic plates were smaller than those formed on the anaerobic plates, and failed to grow aerobically upon a second transfer onto fresh plates.

Manganese chloride can rescue the aerobic growth of the sodA mutant strain (KO2-4)

Not all lactic acid bacteria rely on endogenous superoxide dismutase as a protective mechanism against oxidative stress. *L. plantarum*, that lacks SOD, utilizes stoichiometric amounts of intracellular manganese to scavenge O_2^- and survive in aerobic environment [20,24]. The MRS medium commonly used to grow lactobacilli contains $\sim 330 \mu M$ manganese [45]. However, the M17G medium used in this study did not contain significant amounts of manganese. It contained about 1 nM manganese as determined by atomic absorption. To assess whether the addition of extra manganese chloride to M17G would offer protection against oxygen toxicity and oxidative stress in *S. thermophilus* KO2-4, cultures of this *sodA* mutant strain were supplemented with 1 mM $MnCl_2$ and grown under varying

aeration conditions. The addition of 1 mM $MnCl_2$ had no significant effect on the aerobic or the anaerobic growth of the wild-type *S. thermophilus* AO54 (data not shown). Also, anaerobic growth of the mutant was not affected by the presence of $MnCl_2$ (Fig. 5). However, manganese ions were able to significantly improve the growth of *S. thermophilus* KO 2-4 under microaerophilic conditions (still culture), and to a lesser extent under aerobic (200-rpm) conditions (Fig. 5). Thus, the addition of manganese reduced the doubling time of the mutant by 20% (i.e., from 72 to 58 min) and increased the final cell density by 52% (i.e., from OD₆₀₀ 2.94 to 4.47). Under aerobic conditions (200 rpm), the addition of manganese improved the generation time and the final cell density by only 12 and 28%, respectively. However, higher concentrations of manganese were not tested.

Discussion

In this study, the gene encoding for the manganese-containing superoxide dismutase from *S. thermophilus* was cloned and sequenced. This is the first report revealing the complete sequence of *S. thermophilus sodA* (GenBank Accession No. AF538722). Furthermore, the Gram-positive temperature sensitive vector pG⁺host9 was successfully used to construct a *S. thermophilus* strain deficient in SodA. The mutant was hypersensitive to aerobic growth.

The superoxide dismutase gene of *S. thermophilus* was identified on a 4.4 kb DNA fragment from a genomic library of the organism. This fragment contained two complete open reading frames (orfs). The first orf was found to encode for a 344 amino acid protein that showed a high homology (81%) with the delta subunit of DNA polymerase III from the pathogenic streptococci species *S. pyogenes* [39]. The second orf was shown to encode for the *sodA* gene. Interestingly, the physical arrangement of orf1 (encoding the delta subunit of DNA polymerase) and orf2 (encoding SodA) in *S. thermophilus* is similar to that found in *S. agalactiae* [37].

The *sodA* sequence of *S. thermophilus* also showed a high degree of similarity to other *sodA* sequences of streptococci and other prokaryotes (Fig. 2). Moreover, neither the *sodA* gene in the commercially important *S. thermophilus* nor in the pathogen *S. agalactiae* appears to be induced by the presence of oxygen. Thus under the growth conditions used, the *sodA* gene in both of these organisms seems to be constitutively expressed [21,37]. This is unique given the fact that the majority of the manganese superoxide dismutases found in most other prokaryotes are differentially expressed in the presence or absence of oxygen [12]. However, it is possible that oxygen may induce other aspects of the antioxidant defense(s) in these organisms.

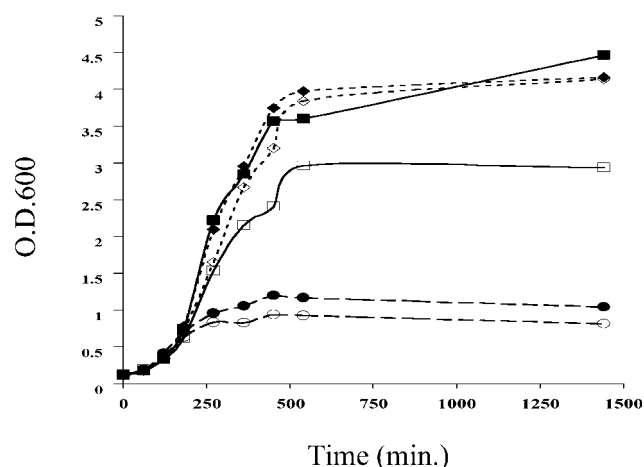


Fig. 5. Effect of $MnCl_2$ on the growth of *S. thermophilus* Strain KO2-4 under different conditions. *S. thermophilus* (KO2-4) cultures were grown at 42 °C in M17G broth supplemented with (closed symbols) or without (open symbols) 1 mM $MnCl_2$. Symbols: (◆, ◇), anaerobic growth; (■, □), aerobic static growth; and (●, ○), aerobic shaking at 200 rpm. The experiment was repeated twice with similar results. [Optical densities at 600 nm were plotted versus time to show the actual changes in the OD₆₀₀ values (i.e., without logarithmic transformation of the OD₆₀₀).]

The *sodA* gene of *S. thermophilus* was functional in the SodA SodB deficient strain of *E. coli* (NC906) and was capable of rescuing its paraquat-sensitive phenotype [27]. The activity of MnSOD expressed from pSODA-4.4 in the *sodAsodB* mutant of *E. coli* (NC906) was affected by the degree of aeration (Table 2). This could be due to the effect of the faster growth rate on the plasmid copy number or on the intracellular concentration of manganese ions, required for the activity of MnSOD. No attempt was made to differentiate between these two or other possibilities. Indeed, further studies are needed to understand the nature of this phenomenon.

In analyzing the upstream sequence of the *sodA* gene, a conserved streptococcal ribosomal binding site could be detected (Fig. 3). However, the predicted –10 and –35 sites (Fig. 3B and GenBank Accession No. AF538722) were not similar to other –10 and –35 sequences from *S. thermophilus* that have been identified previously [46,47]. Interestingly, the –35 sequence appears to be flanked by an inverted repeat, a potential site for regulation. This is consistent with the –35 sequences of *sodA* genes identified in *L. lactis* and *E. coli* [19,43]. It should be noted that currently there is a relatively small body of work on promoter regions in *S. thermophilus*, and those sequences that have been identified vary greatly from one promoter region to the next [46]. The *sodA* gene from *S. agalactiae* similarly did not contain consensus –10 and –35 sequences [37]. Primer extension analysis (Fig. 3) positioned the transcriptional start site of *sodA* of *S. thermophilus* at approximately 27 nucleotides upstream from the start codon. This position is different from that reported for *sodA* from *S. agalactiae* which is 256 nucleotides upstream from the start codon [37]. Additional work is needed to characterize promoter elements in lactic acid bacteria.

It should be noted that *S. thermophilus* possesses only one form of SOD [21], and the disruption of the gene encoding for this essential antioxidant enzyme would result in a SOD-null strain that is very sensitive to oxygen. Indeed, the *sodA* mutant of *S. thermophilus* was more sensitive to growth under aerobic conditions than its parent strain, and exhibited a dramatic decrease in viability when the culture was plated under anaerobic conditions, then the plates were removed from the anaerobic chamber and incubated aerobically (Table 3). Previous studies have shown that organisms lacking superoxide dismutase are more susceptible to oxidative stress. For example, *E. coli* cells deficient in SodA and SodB are unable to grow aerobically on glucose-minimal medium [27] and are hypersensitive to the superoxide generating compound paraquat. SOD deficient strains of yeast are also more sensitive to oxygen and paraquat [48,49]. Unfortunately, we were unable to study the effect of paraquat on the mutant or the parent strains of *S. thermophilus*, due to the inability of the mutant to grow in air and the inability of the parent strain to

transport paraquat or other redox active compounds (unpublished results, [21]).

In this study, it was shown that the presence of manganese in the growth media was able to significantly improve the microaerophilic (still culture) growth of the *sodA* deficient *S. thermophilus* (Fig. 5). Under aerobic growth conditions (shake culture), the addition of MnCl₂ (1 mM) slightly improved the growth of the mutant; however, higher concentrations were not tested. Exogenous MnCl₂ was also unable to improve growth of the wild-type *S. thermophilus* strain under aerobic or anaerobic conditions. Previous studies have shown that other lactic acid bacteria that lack SODs may use stoichiometric amounts of manganese ions for protection against oxygen toxicity [20,24]. At the present time, the physiological contribution of manganese ions in the protection of *S. thermophilus* AO54 against oxygen toxicity in its natural environment (i.e., dairy products) is not known.

In conclusion, the *sodA* gene product (MnSOD) is essential for the aerobic growth of *S. thermophilus* AO54 and assists in dealing with oxidative stresses that may be encountered by this organism under normal growth conditions.

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