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Characterization of Superoxide Dismutase in *Streptococcus thermophilus*

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***Streptococcus thermophilus* AO54 possesses a single manganese-containing superoxide dismutase (MnSOD). The enzyme was found to be insensitive to cyanide or to a modified H_2O_2 treatment. The enzyme is expressed in a growth-phase-dependent fashion, increasing three- to fourfold upon entry into stationary phase. The specific activity for MnSOD was the same under anaerobic or aerobic conditions and was not induced by the presence of paraquat under aerobic conditions.**

Streptococcus thermophilus, a gram-positive facultative anaerobe, represents a commercially important lactic acid bacterium predominantly used as a starter culture (39). In general, lactic acid bacteria (LAB) do not require strict anaerobic growth conditions, and the normal production process lends itself to oxygen exposure. Further, LAB are known to uptake oxygen from their environment at substantial rates in the presence of oxidizable substrates such as NADH and pyruvate (7, 10, 11). Consequently, this leads to the production of partially reduced oxygen intermediates: superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) (17). In addition, the direct reduction of oxygen to H_2O_2 in LAB results largely from the activity of NADH:H_2O_2 oxidases (1, 15, 38). Fortunately, cells have evolved mechanisms to defend themselves against oxygen toxicity via antioxidant enzymes (9, 17, 21) such as superoxide dismutases (SODs) and hydroperoxidases, which scavenge $O_2^{\cdot-}$ and hydrogen peroxide, respectively.

SODs (EC 1.15.1.1) are metalloenzymes which catalyze the dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 (27). There are three types of SODs that are distinguished according to their catalytic metal cofactor: copper, manganese, or iron (17). SODs are essential for aerobic survival and are ubiquitous among aerobic and aerotolerant organisms (17) and even some anaerobic organisms (22, 31).

Some LAB are known to have low levels of SOD while others do not. *Lactobacillus plantarum* and several other aerotolerant LAB have evolved a nonenzymatic dismutation system based on the accumulation of high intracellular levels of Mn^{2+} (2, 10), which can stoichiometrically remove $O_2^{\cdot-}$ (2, 3). To the best of our knowledge, there are no published reports on SOD in the thermophilic streptococci. In this study, we report the presence of a single constitutive manganese-containing SOD in *S. thermophilus* AO54.

Growth conditions. Although *S. thermophilus* was at one time considered to be a subspecies of *Streptococcus salivarius*, it is now considered to be a distinct streptococcal species (34). The industrial strain AO54 (30) was chosen for this study. All AO54 cultures were grown in M17 (37) supplemented with 0.5% glucose (M17G) at 39°C. Cultures were inoculated (0.5% inoculum) with an overnight culture of AO54. Anaerobic growth conditions were maintained in a Coy anaerobic chamber. Aerobically grown cultures were grown with shaking at 150 rpm or without shaking (still culture). The culture/flask ratio was 1/5 for all aerobically grown cultures. For metal supplementation studies, the Chelex-treated medium was the same as M17G except that it was treated with Chelex-100 (Bio-Rad Laboratories) to chelate heavy metals. One millimolar $MgSO_4$ was added to Chelex-treated medium to enhance growth, and ultrapure manganese, iron, or both (atomic absorption standards; Fisher Scientific, Fair Lawn, N.J.) were added as needed.

Assays. Cultures of AO54 were harvested by centrifugation at 4°C at 10,000 × *g* for 5 min, washed in 0.05 M potassium phosphate buffer containing 0.1 mM EDTA (pH 7.8; KPi-EDTA buffer), pelleted by centrifugation as before, resuspended in KPi-EDTA buffer, and disrupted either by sonication for a 30-s burst for a total of 10 min with a Heat Systems W-370 sonicator or by bead beating for 6 min with a MiniBeadbeater-8 (Biospec Products, Bartlesville, Okla.). Overheating was prevented by placing the tubes in an NaCl-ice slurry for ~2 min between the sonication cycles. Cellular debris was removed by centrifugation at 17,000 × *g*. Cell extracts (CFEs) were dialyzed against three changes of KPi-EDTA buffer for a total of 24 h and finally centrifuged at 4°C at 27,000 × *g* for 10 min. The protein concentration was determined by the method of Lowry et al. (25) with bovine serum albumin as the standard.

SOD was assayed by the cytochrome *c* method (27). Potential SOD isozymes were separated on 10% nondenaturing polyacrylamide gels (8) and stained for SOD activity with nitroblue tetrazolium (NBT) (5). To identify the type of SOD, duplicate gels were incubated with 2 mM KCN or 15 mM H_2O_2 during activity staining to inactivate CuZnSOD or FeSOD, respectively (4, 6).

***S. thermophilus* contains MnSOD.** CFEs from aerobically grown *S. thermophilus* cultures were prepared and assayed for total SOD activity (27). Staining for activity (5) revealed a single band that was not inhibited by CN^- (data not shown), thus ruling out the possibility of a CuZnSOD. However, the presence of H_2O_2 (15 mM) in the NBT staining solution resulted in what appeared to be partial inhibition (~50%) of the SOD activity (Fig. 1). We hypothesized that AO54 might represent another example of the rare class of cambialistic SODs such as those found in *Streptococcus mutans* (26), *Bacteroides fragilis* (12, 13), and *Propionibacterium shermanii* (28). To rule out the possibility of an FeSOD that has a peculiar resistance to inhibition by hydrogen peroxide, gels were treated with increasing concentrations of H_2O_2 (up to 300 mM). Despite the use of as much as 300 mM H_2O_2, the SOD was not completely inactivated (data not shown). Similar experiments, in which the CFEs were pretreated with H_2O_2 and then the

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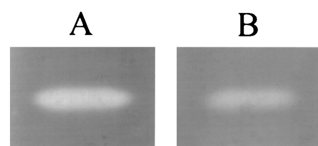

FIG. 1. Effect of H_2O_2 on the activity of SOD in CFEs of *S. thermophilus*. CFEs were prepared and electrophoresed (45 μg of protein/lane) on 10% polyacrylamide gels in duplicate. (A) No treatment; (B) treatment with 15 mM H_2O_2.

excess H_2O_2 was removed by dialysis before assaying for SOD by the cytochrome *c* method (27), were carried out in solution. We expected that, if the enzyme was indeed a cambialistic SOD, then the specific activity of the H_2O_2-treated samples would be about half that of the untreated samples. This was not the case. In this experiment, cells were grown aerobically with shaking at 150 rpm to early stationary phase. CFEs were prepared and dialyzed, and CFE aliquots were treated for 1 h with various concentrations of H_2O_2. The H_2O_2-treated extracts were dialyzed against KPi-EDTA buffer for 12 h to remove H_2O_2 before the assay for SOD. SOD concentrations were found to be as follows: for 0, 15, and 30 mM H_2O_2, 63.8, 62.8, and 61.7 U/mg, respectively. Thus, we concluded that the partial inhibition seen by the activity staining method was due to an unreported interference by H_2O_2 in the NBT assay. We therefore devised a modified protocol for the activity staining procedure in which the gels were preincubated with H_2O_2 (which irreversibly inactivates FeSOD [4] but not MnSOD) and then washed with buffer and treated with bovine catalase (50 U/ml) to remove any residual H_2O_2 before staining for activity with NBT (5). This modified procedure resulted in no partial inhibition of the enzyme (compare Fig. 2 and Fig. 1). Therefore, *S. thermophilus* AO54 possesses a manganese-containing enzyme, MnSOD.

In order to provide additional evidence for the presence of an MnSOD in *S. thermophilus* AO54, the metals in M17G medium were removed by Chelex-100, and then the medium was supplemented with various concentrations of either manganese, iron, or both. Similar experiments were performed with non-Chelex-treated M17G medium. The MnSODs and

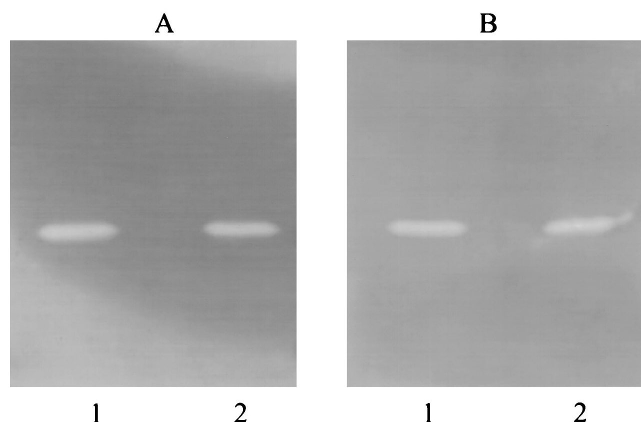

FIG. 2. Effect of modified H_2O_2 treatment on the activity stain for SOD in different CFEs of *S. thermophilus* AO54. Dialyzed CFEs were prepared and electrophoresed (45 μg of protein/lane) as for Fig. 1. Gel A was treated with H_2O_2 (15 mM in KPi-EDTA buffer) for 30 min, quickly rinsed with water, and incubated for 30 min in the KPi-EDTA buffer containing 50-U/ml catalase to remove any residual H_2O_2, while gel B was treated the same way without the addition of H_2O_2. Extracts were prepared from cells grown under the following conditions: aerobic conditions and shaking (150 rpm) (lanes 1) and aerobic conditions and shaking in the presence of 0.1 mM paraquat (lanes 2).

TABLE 1. Effects of supplementation by Mn or Fe on expression of SOD in *S. thermophilus* AO54[a]

Medium	Metal supplement	SOD (U/mg) ± SD
M17G	None	42.2 ± 7.8
	0.1 mM Mn	46.6 ± 10.8
	0.5 mM Fe	4.5 ± 4.0
	0.3 mM Fe	7.2 ± 5.2
	0.1 mM Fe	17.6 ± 1.7
	0.05 mM Fe	22.5 ± 1.4
	0.1 mM Fe + 0.1 mM Mn	31.4 ± 2.4
Chelated	None	31.8 ± 12.5
	0.1 mM Mn	38.9 ± 13.5
	0.1 mM Fe	8.2 ± 3.8
	0.1 mM Fe + 0.1 mM Mn	23.9 ± 9.0

[a] The chelated medium was the same as M17G except that it was treated with Chelex-100 to remove heavy metals. A total of 1 mM $MgSO_4$ was added to both M17G and chelated M17G media to enhance growth. Ultrapure Mn and Fe were used. Cells were grown aerobically without shaking to early stationary phase. Dialyzed CFEs were prepared and assayed for SOD. Values are based on two to four independent experiments.

FeSODs are known to exhibit strict metal cofactor specificity (23). Substitution of Fe for Mn, or vice versa, in *Escherichia coli* renders the enzyme inactive (32, 33). Therefore, we postulated that, if the SOD is indeed an MnSOD, then supplementation by iron or removal of the metals by chelation would lead to the formation of an inactive enzyme due to the presence of the wrong metal (Fe) or no metal at all (apoprotein), respectively. Indeed, the addition of iron to M17G and Chelex-treated M17G medium resulted in diminished SOD activity in *S. thermophilus*; however, this was not the case when Mn was added (Table 1). An inverse relationship between the concentration of iron and SOD activity was observed. For example, the addition of 0.5 mM Fe to M17G caused ~90% inhibition of SOD activity, while 0.05 mM Fe caused a ~50% inhibition (Table 1). Furthermore, 0.1 mM Mn had no effect on SOD activity in M17G and slightly increased the activity in the chelated medium (presumably due to the augmented availability of the correct metal), whereas the same concentration of Fe reduced the activity by more than 50% in both chelated and unchelated media (Table 1). This inhibition by iron could be reversed by supplementing the medium with an equimolar concentration of manganese; thus manganese clearly outcompetes iron for insertion into the active site of the nascent protein (Table 1). These data clearly indicate that the SOD of *S. thermophilus* is a manganese-containing enzyme. Attempts were made to replace the metal via denaturation-renaturation (23), but the recovery was less than 5% (data not shown).

Effects of oxygen and paraquat. MnSOD in *E. coli* (14, 18) and *Lactococcus lactis* (16) is known to be induced by oxygen. To determine if the expression of MnSOD in *S. thermophilus* was affected by oxygen, cultures were grown under anaerobic and aerobic (with and without shaking) conditions and harvested at early stationary phase, and CFEs were assayed for SOD. The specific activities of MnSOD in the CFEs of anaerobic, aerobic (still), and aerobic (150 rpm) cultures were 46.8 ± 2.3, 55.3 ± 7.2, and 64.5 ± 1.3, respectively. These data indicate that the specific activity of MnSOD is not significantly affected by oxygen concentration. Although the specific activity under anaerobic conditions was consistently lower than that under aerobic conditions, the difference in activity was not sufficient to conclude that the MnSOD of *S. thermophilus* is induced by oxygen. In *E. coli*, the presence of redox cycling compounds capable of generating $O_2^{\cdot -}$ causes the induction of

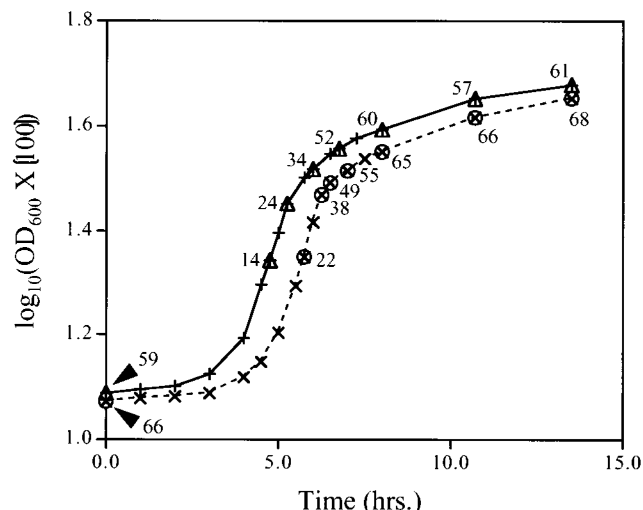

FIG. 3. Effect of growth phase on the aerobic expression of MnSOD in *S. thermophilus* AO54. Cultures were aerobically grown with shaking at 150 rpm (dashed line) or without shaking (solid line) to the indicated optical densities. Dialyzed CFEs were prepared, and protein and SOD were assayed. Curved lines represent growth (optical density at 600 nm [OD_{600}]), and numbers on the curved lines indicate the specific activity of SOD (units per milligram) at the indicated optical density. Triangles and circles indicate the optical densities at which the samples were removed to assay SOD.

MnSOD (19, 20). Therefore, we tested the effect of adding 0.1 mM paraquat on the expression of MnSOD in *S. thermophilus* AO54. The data (Fig. 2, lanes 2) show that paraquat did not induce MnSOD in this organism. Higher concentrations of paraquat (>1 mM) were inhibitory and did not increase MnSOD activity.

Effect of growth phase. Little is known about gene expression during the growth cycle of *S. thermophilus* (29). In a number of bacteria, SOD is induced during stationary phase (35, 36). To determine if the MnSOD levels were affected by the growth phase of the organism, we monitored the expression of SOD throughout the growth cycle. Cultures were grown aerobically, with and without shaking, to the indicated growth phase, aliquots were immediately harvested and dialyzed, CFEs were prepared, and protein and SOD were assayed. SOD (in units per milligram of protein) was found to increase three- to fourfold upon entry into the stationary phase compared to SOD in cells from the exponential phase (Fig. 3).

Conclusion. *S. thermophilus* AO54 contains an MnSOD. The presence of an MnSOD was further supported by metal supplementation studies in which iron was shown to inhibit the activity of the enzyme, presumably by inserting itself into the apoenzyme and thus rendering it inactive. This was not observed when manganese was added to the growth medium. The activity of MnSOD in *S. thermophilus* AO54 was found to be essentially the same under both aerobic and anaerobic conditions and was not induced by paraquat. Furthermore, the specific activity of SOD during the growth cycle of *S. thermophilus* increased three- to fourfold during entry into the stationary phase. We concluded that the MnSOD in AO54 is expressed in a growth-phase-dependent manner. In *E. coli* and in other gram-negative organisms, the expression of growth-phase-dependent genes (i.e., those for catalase, acid tolerance, virulence factors, and antibiotic resistance) is regulated by the stationary-phase sigma factor, σ^S (RpoS) (24). Further studies are therefore necessary to determine whether the MnSOD gene of *S. thermophilus* is regulated by an RpoS homolog and to define the environmental signal required for this response. We are currently cloning the *sodA* gene from *S. thermophilus* and constructing *sodA* null mutants in order to study the physiological role and the regulation of MnSOD in this commercially important organism.

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