Inactivation of Manganese Superoxide Dismutase Diminishes Acid Tolerance in Bacteria

Jose M. Bruno-Bárcena¹,², M. Andrea Azcárate-Peri³, and Hosni M. Hassan¹*

¹Departments of Microbiology, ²Golden Leaf Bio-manufacturing Training and Education Center, North Carolina State University, Raleigh, North Carolina 27695-7615; ³Department of Cell and Molecular Physiology School of Medicine at University of North Carolina, Chapel Hill, NC 27599-7545

*To whom correspondence should be addressed:
Email: hosni_hassan@ncsu.edu
Phone (919) 515-7081
Fax (919) 515-7867

Running title: Mn-superoxide dismutase and acid tolerance
ABSTRACT

Growth in aerobic environments has been shown to generate reactive oxygen species (ROS) and to cause oxidative stress in most organisms. Antioxidant enzymes, i.e., superoxide dismutases and hydroperoxidases, and DNA repair mechanisms provide protection against ROS. Acid stress has been shown to be associated with the induction of Mn-Superoxide dismutase (MnSOD) in Lactococcus lactis and Staphylococcus aureus. However, the relationship between acid stress and oxidative stress is not well understood. In the present study, we showed that mutations in the gene coding for MnSOD (sodA) increased the toxicity of lactic acid at pH 3.5 in Streptococcus thermophilus. The inclusion of the iron chelators 2, 2’-dipyridyl (DIP), diethylenetriamine-pentaacetic acid (DTPA), or o-phenanthroline (O-Phe) provided partial protection against 330 mM lactic acid at pH 3.5. The results suggested that acid-stress triggers an iron-mediated oxidative stress that can be ameliorated by MnSOD and iron chelators. These findings were further validated in E. coli strains lacking both MnSOD and FeSOD but expressing a heterologous MnSOD from S. thermophilus. We also found that, in E. coli, FeSOD did not provide the same protection afforded by MnSOD, and that hydroperoxidases are equally important in protecting the cells against acid stress. These findings may explain the ability of some microorganisms to survive better in acidified environments, such as acid foods, during fermentation and accumulation of lactic acid, or during passage through the low pH of the stomach.
**INTRODUCTION**

Superoxide dismutases (SODs; EC 1.15.1.1) are metalloenzymes that catalyze the conversion of the superoxide anion to hydrogen peroxide and dioxygen (41). Four types of SOD have been characterized, which differ in their metal co-factors. One class consists of SODs with copper and zinc (Cu/ZnSOD), another with manganese (MnSOD), a third with iron (FeSOD), and a fourth with nickel (NiSOD) (30, 65). These enzymes are found across a broad range of organisms and each organism can use one or more type of the enzyme to meet their antioxidant needs (30). For example, *Escherichia coli* possesses three isoforms, MnSOD, FeSOD, and CuZnSOD (9, 34, 64).

Lactic acid bacteria (LAB) are acid-tolerant organisms that require sugar as a source of carbon and energy, generating mainly lactate as a final fermentation product. In particular, the homofermentative *Streptococcus thermophilus*, either alone or together with other species, is extensively employed in the production of yoghurt and other dairy products in which acidification guarantees preservation. LAB are constantly faced with environmental conditions that can affect their growth and viability. Two of the major threats are: acid stress caused by organic acids generated during the fermentation process, and oxidative stress caused by the generation of reactive oxygen species (ROS) during growth in presence of oxygen.

The majority of the LAB possess an inducible acid tolerance response (ATR), also known as acid adaptive response, which improves the survival of adapted cells upon exposure to a lethal acid challenge as compared to the unadapted cells. The induction of the ATR often protects the cells not only from acid challenge, but also from other stresses (18, 24, 60). Regarding the oxidative stress, LAB are classified as catalase (hydroperoxidases) negative and microaerophilic (7). They lack a functional electron transport chain, but they can grow in the presence of molecular oxygen.
However, they contain enzymes that use oxygen such as pyruvate oxidases (17, 51, 52), NADH oxidases that produce \( \text{H}_2\text{O}_2 \), and NADH peroxidases able to break down peroxides (17, 26, 33, 37, 56, 57). Consequently, they generate ROS during their growth in aerobic environments. To offset the harmful effects of ROS, some LAB have evolved protective mechanisms that utilize antioxidant enzymes, such as superoxide dismutases and hydroperoxidases (i.e., catalases and peroxidases; or KatE and KatG), which scavenge superoxide radicals and hydrogen peroxide, respectively, and thus, prevent the formation of \( \text{HO}^\cdot \) via Fenton chemistry (21).

In most *Streptococcus* and *Lactococcus* spp. elimination of ROS conforms to this general antioxidant defence system since both genera possess MnSOD (44, 48), however, they lack of hydroperoxidases. Instead of using SOD, *Lactobacillus plantarum* developed an alternative non-enzymatic defence system that involves the accumulation of high intracellular concentrations of manganese ions, which can scavenge \( \text{O}_2^- \) (4). Previous work has shown that *S. thermophilus* possesses only one type of SOD, the Mn-containing enzyme (MnSOD) (3, 15, 44). The gene encoding MnSOD (sodA) from *S. thermophilus* AO54 has been characterized, cloned, and heterologously expressed in other bacteria (3, 13, 14). Unlike most sodA genes, the *S. thermophilus* sodA is constitutively expressed and is not induced by oxygen or the redox cycling compound, paraquat (15). This antioxidant enzyme (MnSOD) was shown to be essential for the aerobic growth of *S. thermophilus* (3). Consequently, the activity of MnSOD was found to increase in a growth dependent fashion, increasing three- to four-fold upon entry into stationary phase (15), which may be related to regulation of manganese transport (32).

Stress responses are complicated processes that involve the synthesis of a variety of proteins (8, 61). Only a few of the putative acid-resistance proteins have been
characterized (8, 18, 60). Here we present evidence showing that MnSOD provides protection against acid stress. A plausible mechanism is proposed to explain the relationship between acid stress and oxidative stress and how MnSOD confers a survival advantage against both types of stress.

MATERIALS AND METHODS

Bacterial strains and media
The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown at 37 °C in Luria-Bertani (LB) medium (47) supplemented with the appropriate antibiotics. The antibiotics used were: chloramphenicol (Cm, 20 µg/ml), kanamycin (Km, 50 µg/ml), Ampicillin (Amp, 100 µg/ml), and erythromycin (Em, 200 µg/ml).

S. thermophilus AO54 (43) was grown at 37°C in Difco™ Lactobacilli MRS broth (19). When required, erythromycin (2 µg/ml) was added to S. thermophilus cultures. Solid media for plating were prepared by adding 1.5% agar to the appropriate liquid media.

Bacterial transformations. E. coli strains were transformed via electroporation using a BioRad Gene Pulser (BioRad, Richmond, CA) according to the manufacturer instructions.

Sources of Chemicals and enzymes. DL-lactic acid (89%), 2, 2’- Dipyridyl (DIP), Diethylenetriamine-pentaacetic acid (DTPA), O-Phenanthroline (O-Phe), and all antibiotics used were purchased from Sigma (St. Louis, MO). All other chemicals and the bacteriological media were purchased from Fisher Scientific (Pittsburgh, PA).
Acid Tolerance Studies. To prepare exponentially growing cells of *S. thermophilus* or *E. coli*, overnight cultures were used to inoculate appropriate media to an initial OD$_{600}$ of 0.05. Cells were allowed to grow at 37°C and changes in OD$_{600}$ were monitored over time until the cultures reached OD$_{600}$ = 0.2 to 0.4. The cells were then harvested by centrifugation, washed, and re-suspended in the same media pre-acidified with DL-lactic acid (330 mM, pH 3.5).

In the acid pre-adaptation experiments, cells were incubated for 30 min in the appropriate media pre-acidified with DL-lactic acid (33 mM, pH 5.5) before they were subsequently challenged with DL-lactic acid (330 mM, pH 3.5) as described above. Aliquots of the cell culture were withdrawn at various time intervals (0, 30, 60, 90, and 120 min), diluted in the non-acidified media (pH 6.5–7.0), and spread on LB or MRS agar plates. Plates were incubated at 37°C and counted (cfu/mL) after 24 h for *E. coli* or 48 h for *S. thermophilus*.

Effect of Iron Chelators. Increasing concentrations of the iron chelators (2, 2’-Dipyridyl, Diethylenetriamine-pentaacetic acid, and O-Phenanthroline) were added to exponentially growing *S. thermophilus* during acid challenge in MRS media containing 330 mM DL-lactic acid, pH 3.5 at 37°C. At different time intervals 10 µL aliquots were removed and spotted on solid media and incubated at 37°C.

Metal Analyses. Overnight culture of *S. thermophilus* AO54 was used to inoculate MRS medium to an initial OD$_{600}$ of 0.05. Cells were grown to an OD$_{600}$ = 1 before they were harvested by centrifugation, washed in sterile media, and subsequently challenged in 20 mL of MRS pre-acidified with DL-lactic acid (330 mM, pH 3.5) or HCl (330 mM, pH 3.5) for 30 min. At the end of the acid challenge, cells were harvested, washed with...
**RESULTS**

*Can MnSOD Protect S. thermophilus Against Acid Stress?*

Acid-stress has been suggested to lead to oxidative stress (16, 48); however, conclusive relationship between the two stresses is lacking. In this study, we hypothesized that antioxidant enzymes like superoxide dismutases (SODs) and hydroperoxidases must have a role in protecting the cells against acid-stress. Thus, we evaluated the physiological role of MnSOD in protecting *S. thermophilus* against acid-stress. We used both acid-adapted and non-adapted cells from exponentially growing cultures of the wild-type *S. thermophilus* (AO54) which contains MnSOD gene (*sodA*) and its isogenic ∆sodA strain (KO2-4) which lacks MnSOD (Figure 1). Non-adapted cells were directly exposed to DL-lactic acid (330 mM, pH 3.5), while adapted cells were first incubated in a non-lethal concentration of DL-lactic acid (33 mM and pH 5.5) to trigger the acid tolerance response (ATR) prior to exposure to DL-Lactic acid (330 mM, pH 3.5). By using both adapted and non-adapted cells, we were able to differentiate between resistance to acid stress and the ATR response.

Figure 1 shows that exponential-phase cells of *S. thermophilus* KO2-4 (∆sodA) were more sensitive to lactic acid acidified medium (pH 3.5) as compared to its isogenic wild-type strain. Data in Figure 1 also show that both wild-type (WT) and ∆sodA
cultures were able to mount an adaptive ATR. However, in adapted cells, the mutant was still more sensitive to DL-Lactic acid (330 mM, pH 3.5) than the WT (compare solid circles and solid squares in Fig.1).

**Is Lactic Acid Toxicity Related to Iron-Mediated Fenton Chemistry?**

In this part of the study, we examined the effect of added lactate or HCl on the intracellular levels of iron in *S. thermophilus* AO54. As described in materials and methods, the cells were grown, collected, and exposed to pre-acidified (i.e., 330 mM DL-lactic acid, pH 3.5) MRS medium. The MRS medium used contained 10.56 µM of total iron (i.e., 0.59 µg/mL). The concentration of iron in cells exposed to either lactate or HCl were 3.09±0.8 ng and 3.68±0.4 ng iron/ OD$_{600}$, respectively; while the iron content in untreated cells was 4.02±0.9 ng of Iron/OD$_{600}$. It is clear that total intracellular iron content of acid exposed cells was not significantly different from that of the untreated control cells. These results, however, do not differentiate between free and bound iron in the cells. It has been shown that lactate stimulates fibroblast proliferation (62), wound healing (59), enhances iron bioavailability in foods (45), and increases iron absorption by the human colon carcinoma cell line (Caco-2 cells) (10). Furthermore, lactic acid has been shown to chelate Fe$^{3+}$ in a 1:1 ratio (29) and that lactate-iron complex can generate hydroxyl radicals (2). Therefore, we decided to verify the involvement of iron and Fenton chemistry in lactate toxicity by examining the effects of iron chelators on acid toxicity. We employed chelators that are known to be able to chelate intracellular iron [2, 2’-dipyridyl (DIP), diethylenetriamine-pentaacetic acid (DTPA), and O-Phenanthroline (O-Phe)]. Data in figure 2 indicate that the removal of intracellular free iron provided partial protection against lactate toxicity. It should be noted that the...
permeability as well as the iron binding affinity of the chelators used in this study are, most likely, not identical. Therefore, the results in Figure 2 qualitatively demonstrate the involvement of free intracellular iron in the toxicity of lactic acid under the experimental conditions employed.

Can Heterologous MnSOD Protect sodA sodB mutant of E. coli Against Acid Stress?

To further corroborate the contribution of MnSOD to acid resistance, we used the sodA gene from S. thermophilus to complement E. coli NC906, a strain with deletions in both of the endogenous Mn-and FeSOD genes (sodA sodB). Data in Figure 3 show that the heterologous MnSOD was able to protect E. coli NC906 cells against acid stress.

Can FeSOD Protect E. coli Against Acid Stress?

The Mn- and Fe-SODs of E. coli are highly homologous, and the coordination of the metals in the active sites are nearly identical (22). Both enzymes are equally important in protecting E. coli against oxygen toxicity (25, 30, 49). Therefore, it is expected that FeSOD would have the same role as MnSOD in protecting E. coli against acid stress. For this part of the study, we used an E. coli strain (UM2) deficient in katG and katE (38), since previous studies have found the accumulation of weak acids induces catalase expression (50). Thus, we compared the roles of FeSOD and MnSOD in acid stress using isogenic UM2 strains harbouring mutations in sodA, sodB, or both (49) (Table 1).

Figure (4) shows that strains lacking sodA (UM2A) or both sodA and sodB (UM2AB) were more sensitive to acid stress than the parent (UM2) strain. By contrast,
the loss of sodB (UM2B) did not result in a greater sensitivity to lactic acid than that seen in the SOD proficient strain (UM2). However, cells lacking both sodA sodB (UM2AB) were more sensitive to lactic acid than cells lacking only sodA (UM2A).

These data demonstrated that FeSOD is not as efficient as MnSOD in protecting the cells against acid stress.

Can Hydroperoxidases Protect E. coli Against Acid Stress?

Data in figures 3 and 4B (Compare the lines for pre-adapted NC 906 and UM2) show that deficiency of SODs (NC 906) or hydroperoxidases (UM2) resulted in equal losses in viable counts after 120 min of exposure to 330 mM lactic acid at pH 3.5 (i.e., a loss of 2.7 vs. 3.0 logs, respectively). The data clearly suggest that both SODs and hydroperoxidases are important in protecting the cells against acid stress.

DISCUSSION

The ability of microorganisms to adapt and survive high acid/ low pH conditions is essential for their viability in acid foods and/or during passage through the acidic environment of the stomach. This adaptive ability is essential for both beneficial and pathogenic organisms. Exposure to mild acidic conditions triggers an adaptive response also called the acid tolerance response (ATR), where the cells adjust the expression of several genes required for survival in the hostile high acid environment. Proteins whose expression is increased during ATR include F₁F₀-ATPase proton pumps, membrane proteins, DNA and protein repair enzymes, etc. Indeed, the role of acid pH in inducing the proton-translocation F₁F₀-ATPase operon has been demonstrated in both Gram⁺ and Gram⁻ organisms (24, 36, 39).
Mn-superoxide dismutase and acid tolerance

MnSOD has been shown to be induced under low pH conditions in *Lactococcus lactis* (48), *Streptococcus mutans* (53), and *S. aureus* (16). Additionally, accumulation of weak acids in the culture media has been shown to induce the catalase genes in *E. coli* (50) and induce both catalase and superoxide dismutase in *Listeria monocytogenes* (20). Alignment of amino acid sequences of MnSODs from different prokaryotes and eukaryotes show high degree of homology, a highly conserved active center (22), and a conserved catalytic function(s) (i.e., to disproportionate O$_2^-$ to H$_2$O$_2$ and O$_2$) (41). The role of MnSOD in protecting cells against oxidative stress is widely understood and accepted (25). However, its role in protecting the cells against acid-stress has yet to be elucidated.

In this study, we tested the hypothesis that MnSODs also protect cells against acid stress and that free iron plays an important role in cell death during acid exposure. Our data showed that exponential-phase cells from *sodA* mutant strains of *S. thermophilus* and *E. coli* (i.e., KO2-4 and NC906) were less tolerant to pH 3.5 than their isogenic counterparts expressing *sodA* (Figs. 1 and 3). Furthermore, the data showed that MnSOD, regardless of the origin of the *sodA* gene, has a role in protecting *E. coli* against acid stress (Figs. 3 & 4). We also demonstrated that the addition of iron chelators (Fig. 2), and the presence of hydroperoxidases (KatG and KatE) (Figs 3 and 4) provided significant protection against acid toxicity. Taken together, these findings strongly suggest that acid toxicity is mediated by the greater availability of free iron that can react with the partially reduced oxygen species (O$_2^-$ and H$_2$O$_2$) to cause the generation of the damaging hydroxyl radical (HO$^\cdot$).

It was interesting and unexpected to discover that FeSOD was not as effective in protecting *E. coli* against acid stress (Fig. 4). Thus, the loss of *sodA* (UM2A), in acid pre-adapted cells (Fig 4B), resulted in 7 logs reduction in cell viability after 120 min of
acid challenge, while the loss of sodB (UM2B) resulted in ~ 2.9 logs of reduction, which is similar to that seen in the SOD competent cells (i.e., 3 logs reduction in UM2).

This finding is best explained by the fact that iron-containing SODs are inactivated by 
H₂O₂ (6, 11, 12). The finding that FeSOD was less efficient than MnSOD in protecting 
E. coli against acid stress also enforces the conclusion that acid stress is mediated by 
iron catalyzed Fenton chemistry. Furthermore, since hydroperoxidases remove H₂O₂, it 
is not surprising that UM2 cells lacking of both KatG and KatE enzymes were as 
sensitive to lactic acid challenge as those cells lacking SODs (NC 906) (Fig 3 and 4).

Indeed, a recent report has shown that overexpression of catalase reduces lactic acid-
induced oxidative stress in Saccharomyces cerevisiae (1). Furthermore, the co-
induction of catalase and superoxide dismutase by the accumulation of weak acids (20, 
50), supports the notion that acid stress and oxidative stress are related phenomena.

Hydrogen peroxide and superoxide radical are normally generated during 
growth in aerobic and micro-aerobic environments. The deleterious effects of H₂O₂ on 
growth and cell survival have been shown to be dependent on the availability of free 
intracellular soluble iron [Fe²⁺] (23, 35). Lactic acid has been found to increase the 
dissociation of catalytic iron from proteins (46) provoking the reaction between ferrous 
iron and H₂O₂ to generate the highly reactive hydroxyl radical (HO⁻) via the Fenton 
chemistry (21) or via the Haber-Weiss reaction (27, 40, 54). Furthermore, the Fenton 
reaction has been shown to be optimum at acidic pHs (~pH 3.0) (5). In addition, in 
vitro studies have shown that lactic acid iron complex enhances the generation of HO⁻ 
(2). The data presented here and in the literature indicate that the addition of lactic acid 
increases the availability of “Free Intracellular Iron” which can then participate in the 
generation of HO⁻ that reacts indiscriminately with most of the biological molecules and 
kill the cell.
Previous studies have shown that the endogenous SOD levels control the iron-dependent HO formation when cells were exposed to hydrogen peroxide (13, 14, 42) or due to an iron overload as in the fur mutant of E. coli (58). The continuous generation of HO requires a continuous supply of Fe\(^{2+}\), which can be provided by the labile iron-sulfur [4Fe-4S]\(^{2+}\) clusters or via the reduction of Fe\(^{3+}\) by O\(_2^-\) (13, 15, 31). Previously, we demonstrated that MnSOD provides protection against H\(_2\)O\(_2\) (3, 13) likely by interfering with the generation of HO. Figure 5 is a schematic presentation showing how MnSOD, iron-chelation, or hydroperoxidases could protect the cells from both organic acid (e.g., Lactic acid) and ROS stress.

From this study, we conclude that the cytotoxic effects of acid stress and oxidative stress are remarkably similar; i.e., both involve the generation of hydroxyl radicals. We predict that other antioxidant enzymes (e.g., SodC, Alkyl-hydroperoxide reductase) and iron chelating proteins (e.g., Dps, Dpr) could also have a protective role against acid stress. Interestingly, the expression of dpr in S. mutans was differentially increased by acid exposure (54); and in Salmonella Dps was reported to be regulated by Fur (28) that was shown in an earlier study to be essential for acid resistance (63).

Acidophilic microorganisms, like S. thermophilus, (which can grow under low pH conditions) seem to rely on MnSOD and probably other antioxidants for their survival in acidic environments. Furthermore, expressing MnSOD and/or hydroperoxidases in Lactobacillus spp. that lack these antioxidant enzymes may enhance their ability to survive and resist ROS and acid stress.

ACKNOWLEDGMENTS

Partial support was provided by the N.C. Agricultural Research Service. We would like to thank Matt Evans for his technical assistance and critical reading of this manuscript.
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*NC  North Carolina State University culture collection*
**FIGURE LEGENDS**

**Figure 1.** Response of *S. thermophilus* to lactic acid stress. Un-adapted (open symbols) and adapted (closed symbols) cells of exponentially growing *S. thermophilus* [Parent strain - A054 (□,■) and its isogenic ΔsodA strain - KO2-4 (○,●)] (pre-exposed or not during 30 min to 33 mM DL-lactic acid pH 5.5) were challenged in MRS media containing 330 mM DL-lactic acid (pH 3.5). At specific time intervals samples were diluted and plated on agar media to monitor cell viability. The data are mean of triplicate points.

**Figure 2.** Effect of iron chelators in protecting *S. thermophilus* KO2-4 against lactic acid toxicity. Un-adapted exponentially growing cells of *S. thermophilus* KO2-4 (AO54-ΔsodA) were exposed at 37°C in MRS media containing 330 mM DL-lactic acid – pH 3.5 and in the presence of increasing concentrations of chelators (2, 2’- Dipyridyl, Diethylenetriamine-pentaacetic acid, and O-Phenanthroline ). Ten µL aliquots were removed at 0, 15, and 30 min from the different treatments and spotted on solid media and incubated at 37°C as described in Materials and Methods.

**Figure 3.** Effect of heterologous MnSOD from *S. thermophilus*-AO54 on the survival and adaptative response of sodA sodB mutant of *E. coli* (NC906) exposed to lactic acid stress. Exponentially growing cells (*E. coli* NC906 [△,▲] and NC906+pSODA [○,●]) were pre-exposed to 33 mM DL-Lactic acid pH 5.5 (closed symbols) or not exposed (open symbols). After 30 min of treatment, cells (pre-exposed or not exposed) were resuspended in MRS media containing 330 mM DL-Lactic acid pH 3.5. At specific time intervals samples were diluted and plated on LB Agar media to monitor cell viability. The data are mean of triplicate points.

**Figure 4.** Roles of MnSOD and FeSOD in absence of hydroperoxidases (KatG’ and KatE’) on the survival and adaptative response of exponentially growing *E. coli* exposed to lactic acid stress. (A) Cells were not pre-adapted; (B) cells were pre-adapted by exposure to 33 mM DL-lactic acid pH 5.5 for 30 min. The un-adapted and adapted cells were resuspended in MRS medium containing 330 mM DL-Lactic acid pH 3.5. At specific time intervals samples were diluted and plated on LB Agar media to monitor cell viability. Symbols: □, Parent Kat’ strain (UM2); ●, SodA’ Kat’ (UM2A); ▲,
Manganese superoxide dismutase and acid resistance

SodB• Kat• (UM2B); and ▼, SodA• SodB• Kat• (UM2AB). The data are mean of triplicate points.

**Figure 5.** A schematic presentation showing how MnSOD, iron-chelator, or hydroperoxidases could protect cells against oxidative stress mediated by lactic acid. Reaction (1) shows the oxidation of labile iron-sulfur clusters by O$_2^-$; reaction (2) shows the regeneration of Fe (II) from Fe (III) by the O$_2^-$ [the sum of reactions 2 & 3 is also known as the Haber-Weiss reaction]; reaction (3) shows the generation of HO' by the Fenton chemistry. Protective molecules and/or mechanisms are shown in boxes: MnSOD inhibits reactions 1, 2 & 3; hydroperoxidases also inhibit reaction 3; iron-chelators inhibit reaction 3. Lactic acid provides protons and forms an iron-lactate complex that can enhance the generation of HO'.
Figure 1. Manganese superoxide dismutase and acid resistance.
Manganese superoxide dismutase and acid resistance

Figure 2.

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1

2 Figure 2.
Figure 3. Manganese superoxide dismutase and acid resistance.
1
2 Figure 4.
Manganese superoxide dismutase and acid resistance

Figure 5.2

[Diagram showing the reaction involving MnSOD and the formation of iron-lactate complex and free iron]

Figure 5.
Manganese superoxide dismutase and acid resistance

Reference List


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