Characterization of superoxide dismutase in the rumen bacterium *Streptococcus bovis*

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ABSTRACT: Superoxide dismutase (SOD) isoenzymes of the rumen bacterium *Streptococcus bovis* 4/1 were studied. Native PAGE showed a single band of Mn-SOD, unaffected by 10 mM cyanide or 5 mM hydrogen peroxide under both aerobic and anaerobic growth conditions. When the metals were removed from the growth medium by Chelex 100, the addition of manganese increased enzymatic activity, while addition of iron inhibited SOD activity. Changes in Mn-SOD and glutathione peroxidase (GSHPx) activities evoked by paraquat and increased values of TBARS indicated that these enzymes were not able to sufficiently prevent oxidative stress at given paraquat concentrations.

Keywords: *Streptococcus bovis*; paraquat; superoxide dismutase; glutathione peroxidase

The rumen provides an anaerobic environment where both obligate and facultative anaerobic bacteria compete and survive. Partial reduction of oxygen to water during microbial respiration gives rise to reactive oxygen intermediates, e.g. superoxide radicals, hydrogen peroxide and hydroxyl radicals (Fridovich, 1978; Gregory and Dapper, 1983). Microorganisms have developed efficient enzymatic and nonenzymatic mechanisms to eliminate these toxic and mutagenic reactive oxygen species (Storz *et al.,* 1990). Superoxide is eliminated by dismutation to H_2O_2 catalyzed by superoxide dismutase (Fridovich, 1978) and accumulation of H_2O_2 is prevented by the action of catalases and peroxidases (Hassan and Fridovich, 1978). SODs are essential for aerobic survival and are ubiquitous among aerobic and aerotolerant organisms (Hassan, 1989) and even some anaerobic organisms (Kirby *et al.*, 1980). Fulghum and Worthington (1984) presented data that show that some species of ruminal bacteria contain measurable levels of SOD activity. Four SOD isoenzymes have been discovered in prokaryotic and eukaryotic organisms (Bannister *et al.*, 1987). All prokaryotic organisms so far studied contain Mn-SOD or Fe-SOD. Cu/Zn-SOD is absent except for a few cases (Steinman and Ely, 1990). There has been extensive investigation of the function of SOD in the facultative anaerobe *E. coli*, which has two cytoplasmic SODs, cofactored with either iron (Fe-SOD, encoded by *sod*B) or manganese (Mn-SOD, encoded by *sod*A) as well as a recently characterized periplasmic form of the enzyme cofactored with copper and zinc (Cu/Zn-SOD) (Beyer *et al.*, 1991; Belov and Fridovich, 1994).

Streptococcus bovis has been identified as a causative agent of a variety of diseases in humans, but on the other hand it is a normal inhabitant of the rumen of ruminant animals. DNA-DNA hybridization studies indicate that human and ruminal isolates belong to different DNA homology groups (Whitehead and Cotta, 2000). In this report we examined SOD isoenzymes of ruminal *S. bovis* and their activities in aerobic and anaerobic metabolism, and the influence of the redox-active herbicide, paraquat, on its antioxidant enzyme activity.

MATERIAL AND METHODS

Chemicals

All reagents, of the highest purity, were from Sigma, Merck and Boehringer.

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Bacterial strains and growth conditions

Streptococcus bovis 4/1 (Štyriak *et al.*, 1994) used in this study was isolated from the rumen of sheep and is maintained in our microbe collection. *S. bovis* 4/1 was grown aerobically overnight at 37ºC in Todd–Hewitt broth (TH, Imuna, Slovakia) containing no or 0.5, 1.0 and 2.0 mM paraquat or anaerobically in the same medium under an oxygen-free CO_2 atmosphere (Bryant, 1972) with or without paraquat. In order to provide additional evidence of an MnSOD in *S. bovis* 4/1, the metals in Todd-Hewitt medium were removed by Chelex-100 (Bio-Rad Laboratories), and than the medium was supplemented with ultrapure manganese, iron or both. Similar experiments were performed with non-chelex treated medium. The culture in the early stationary phase was harvested by centrifugation at 10 000 × g for 15 min at 4ºC and cells were washed in potassium phosphate buffer containing 0.l mM EDTA, pH 7.4, pelleted by centrifugation as before and resuspended in the same buffer. They were disrupted by sonication using 30-s bursts for a total of 3 min with 1 min cooling periods between bursts using a MSE Soniprep 150 ultrasonic disintegrator at 4ºC. Cellular debris was removed by centrifugation at 12 000 × g for 15 min, the supernatant was dialysed against a potassium phosphate buffer and used for the enzyme assays.

Enzyme assays

Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by measuring the inhibition of cytochrome c reduction using the xanthine/xanthine oxidase O_2^- generating system at 550 nm (Flohé and Ötting, 1984). One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of cytochrome c reduction under the assay conditions.

SOD isoenzymes were separated on 10% nondenaturing polyacrylamide gels (Laemmli, 1970) and the enzyme activity was visualized as achromatic bands by staining with nitro-blue tetrazolium chloride according to Beauchamp and Fridovich (1971). To identify the isoenzymes of SOD, gels were treated with 10 mM KCN or 5 mM H_2O_2 in a buffer for 30 min to inactivate Cu/Zn-SOD or Fe-SOD respectively (Britton *et al.*, 1978).

Glutathione peroxidase activity (GSHPx, EC 1.11.1.9) was measured by monitoring the oxidation of NADPH at 340 nm as described by Flohé and Günzler (1984) in a coupled assay with glutathione reductase. Cumene hydroperoxide or H_2O_2 were used as substrates. Specific activity was defined as the unit of enzyme activity per mg of protein.

Glutathione reductase (GR, EC 1.6.4.2.) was determined by following the decrease in NADPH absorbance at 340 nm due to GSSG reduction (Pinto *et al.*, 1984).

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. Lipid peroxidation products measured as thiobarbituric acid reactive substances (TBARS) were determined according to Gutteridge (1984).

Statistics

The results are given as means ± SEM of at least three independent determinations in three different batches. Data were analysed using Student's *t*-test with a significance level of $P < 0.05$.

RESULTS

When analysing potential SOD isoenzymes of *S. bovis* by native PAGE, a single band was seen under both aerobic and anaerobic conditions. To identify the type of SOD, the gels were treated with 10 mM cyanide or 5 mM H_2O_2 to inactivate Cu/Zn-SOD or Fe-SOD, respectively (Figure 1). To support the finding that SOD activity was only Mn-SOD, *S. bovis* was grown in a medium suplemented 0.1 mM concentration of either iron, manganese or with both. The addition of 0.1 mM Fe to the both unchelated and chelated growth media resulted in diminished SOD activity in *S. bovis*, however, this was not the case when Mn was added (Figure 2). Furthermore 0.1 mM Mn had no effect on SOD activity in unchelated medium and slightly increased the activity in the chelated medium, presumably due to the augmented availability of the correct metal. The same concentration of Fe reduced the activity by more than 40 percent in both unchelated and chelated media. This inhibition by iron could be reversed by supplementing the medium with an equimolar concentration of manganese.

To determine if the levels of SOD and GSHPx were affected by the growth phase of *S. bovis* we monitored their expression throughout the growth cycle under aerobic conditions. Figure 3 shows that the activities of both enzymes were the highest in the stationary phase and the differences were not significant.

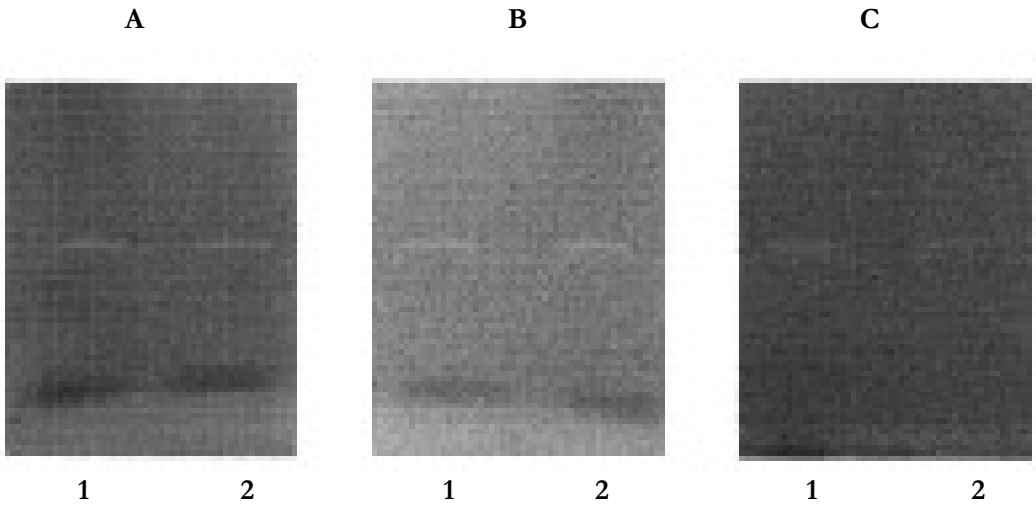

Figure 1. Non-denaturating PAGE analyses of SOD isoenzymes by activity staining. After electrophoresis (100 mg of protein per lane) gels in duplicates were: A – no treatment, B – soaked in 5 mM H_2O_2 for 30 min, C – soaked in 10 mM KCN for 30 min. Then all gels were covered with the solution containing nitro-blue tetrazolium and riboflavin, and exposed to light. Cell extracts were prepared from stationary phase culture of *S. bovis* 4/1 grown under aerobic (1) or anaerobic (2) conditions

The bipyridyl herbicide paraquat increases oxidative stress directly by generating oxygen radicals (Fridovich, 1997). We therefore tested the influence of paraquat on the activities of antioxidant enzymes: SOD, GSHPx (Figure 4) and GR in *S. bovis*. The specific Mn-SOD activities were similar under aerobic and anaerobic conditions (196.4 ± 20.6 and 167.5 ± 14.2 mU/mg protein, respectively). The SOD activity significantly increased in the presence of 1.0 mM paraquat only under aerobic conditions while the anaerobic SOD activity was inhibited. The GSHPx activity determined was with the substrate cumene hydroperoxide, which acts mainly on organic hydroperoxides, and there was no significant difference between the aerobic (3.11 ± 0.09 mU/mg protein) and anaerobic (3.75 ± 0.58 mU per mg protein) activities of controls. The aerobic GSHPx activity was 1.3-fold higher in the presence of 0.5 mM paraquat, the anaerobic activity was not changed. Both aerobic and anaerobic GR activities (54.72 ± 3.02 and 51.81 ± 4.24 mU/mg protein, re-

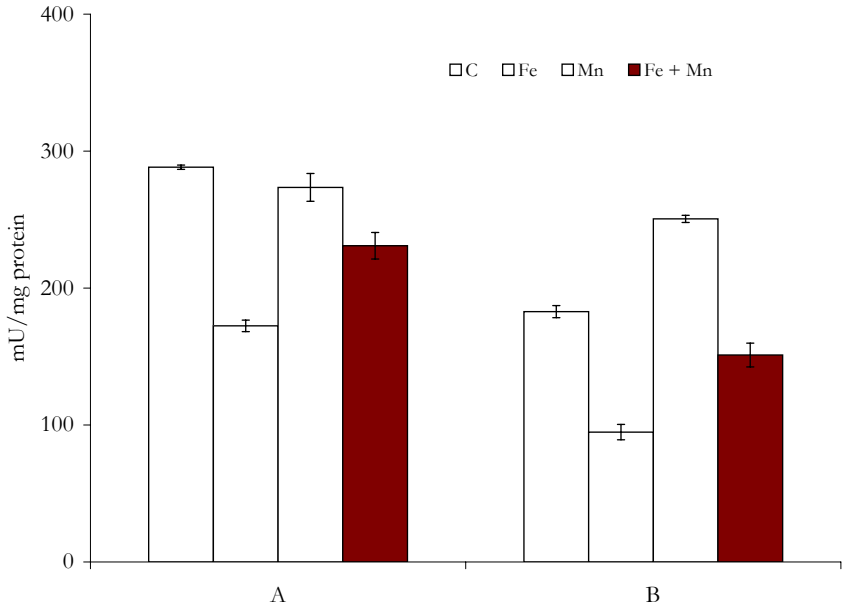

Figure 2. Effects of metals suplementation on expression of SOD in *S. bovis* 4/1. A – bacteria grown in medium supplemented by 0.1 mM concentrations of iron, manganese and 0.1 mM iron + 0.1 mM manganese. B – bacteria grown in a medium chelated by Chelex–100, following the supplementation with the above metals. The conditions are desribed in Material and Mehods

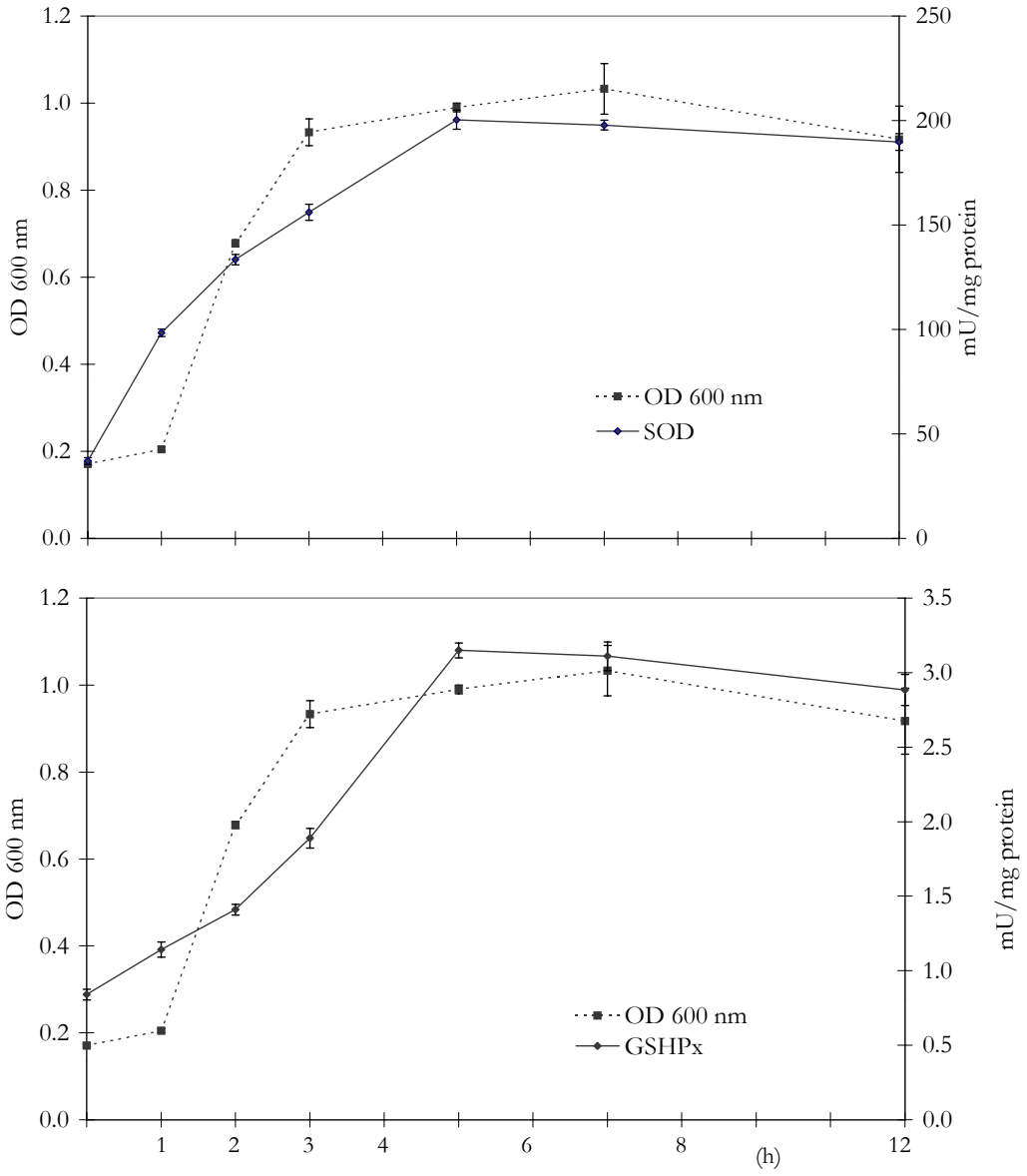

Figure 3. Effect of growth phase on SOD (A) and GSHPx (B) activities of *S. bovis* 4/1. *S. bovis* 4/1 was grown aerobically overnight at 37°C in Todd–Hewitt broth. Growth was monitored as optical density at 600 nm. Samples were withdrawn at time intervals and SOD and GSHPx activities determined as described in Material and Methods. The results are specific activities (units per miligram) of three independent experiments

spectively) were not significantly changed in the presence of 0.5 mM and 1.0 mM paraquat. A higher concentration of paraquat (2.0 mM) was inhibitory for all enzyme acivities. Reactive oxygen species are presumably the agents that cause cellular damage, so we analyzed lipid peroxidation products by determining thiobarbituric acid reactive substances. All concentrations of paraquat evoked significantly increased values of TBARS (Figure 4) which suggests that the antioxidant enzymes SOD and GSHPx were not able to sufficiently prevent the oxidative cell damage.

DISCUSSION

S. bovis belongs to the populations of facultative anaerobic bacteria adhering to the rumen epithelium, which provides "scavenging" activity against oxygen diffusing from the blood through the rumen wall into the rumen (Mead and Jones, 1981). The first line of defence against the generation of toxic oxygen species is SOD. In our experiment the SOD inhibition study to differentiate SOD isoenzymes indicated this activity as Mn-SOD. The SODs are metalloenzymes which

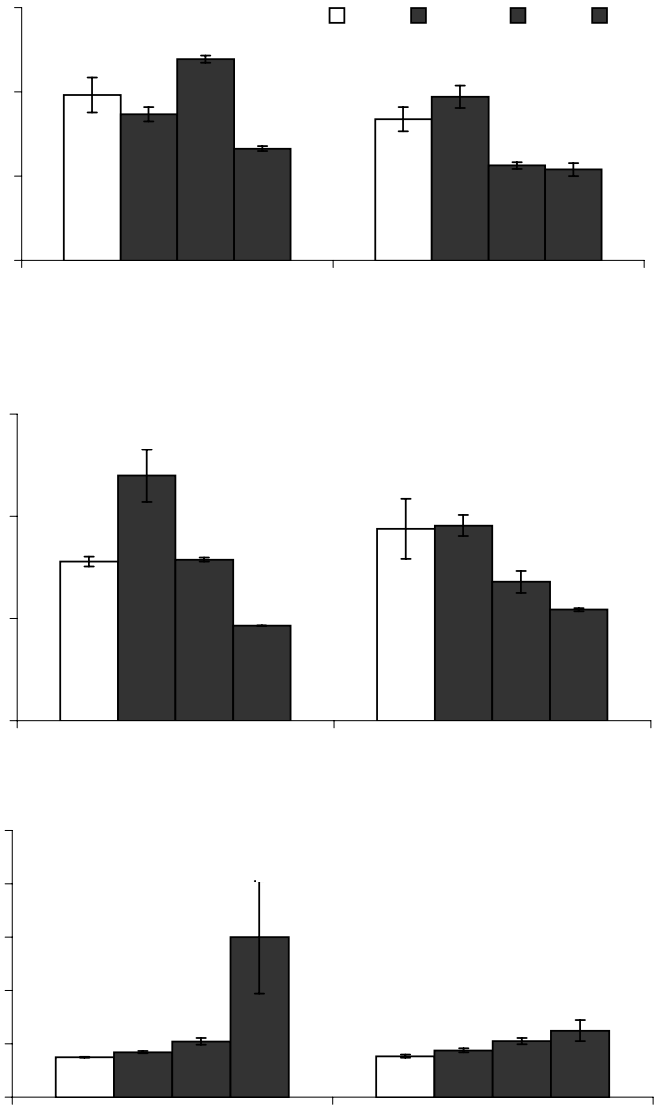

Figure 4. The influence of different concentrations of paraquat on antioxidant enzymes activities and lipid peroxidation products in *S. bovis* 4/1 under aerobic (A) and anaerobic (B) conditions. The specific enzyme activities were measured in mU/mg protein and TBARS content as absorbtion/mg protein

*$P < 0.05$; asterisks represent significant differences between control group (0) and bacteria grown in the presence of paraquat

exhibit strict metal cofactor specificity (Kirby *et al.*, 1980). Substitution of a wrong metal in *E. coli* renders the enzyme inactive (Ose and Fridovich 1976a,b). We postulated that if the SOD of *S. bovis* was indeed an Mn-SOD, the supplementation of the growth medium 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. Indeed, the addition of iron diminished the SOD activity. Manganese and iron play important roles in Mn-SOD biosynthesis. Thus, Mn-SOD activity in *E. coli* can be influenced by the competition of manganese and iron for the nascent SodA protein (apo-SodA) resulting in different forms of the protein: Mn_2-SodA – fully active, Mn/Fe-SodA – partially active and Fe_2-SodA – totally inactive (Hassan and Schrum, 1994). In our experiment, the SOD inhibition of *S. bovis* by iron could be reversed by supplementing the medium with manganese, which

points that manganese outcompetes iron for insertion into the active site of the nascent protein. These results support the idea that the SOD of *S. bovis* possesses a single manganese containing enzyme.

Ruminants can be exposed to the toxic concentrations of pesticides and heavy metals by the ingesting contaminated feed and water. Rumen bacteria may modify their toxicity for the animal. The present study was undertaken to investigate how oxidative stress can influence the antioxidant enzyme activity of the rumen bacterium *S. bovis*. Redox cycling compounds like paraquat capable to generate superoxide radicals cause the induction of Mn-SOD in *E. coli* and also in some other bacteria (Hassan and Fridovich, 1977; Hassan and Fridovich, 1979; Meier *et al.*, 1982), but in contrast, in *S. thermophilus*, a Gram positive, facultative anaerobe, Mn-SOD was not induced, even the high concentrations of paraquat were inhibitory for this activity (Chang and Hassan, 1997).

It is known that Mn-SOD of some bacteria is induced by oxygen (Gregory and Fridovich, 1973). The Mn-SOD activity in ruminal *S. bovis* was not significantly different under the aerobic and anaerobic conditions, therefore we cannot conclude that this activity was induced by oxygen. Our study to determine whether paraquat results in alteration of antioxidant defence enzymes showed that only the aerobic Mn-SOD (1.0 mM paraquat) and GSHPx (0.5 mM paraquat) activities were significantly increased. The increased GSHPx activity of ruminal *S. bovis* was not followed by increasing the GR activity, which is important in maintaining cellular GSH necessary for GSHPx, and prevents the oxidative damage. A higher concentration of paraquat (2 mM) was inhibitory for both the aerobic and anaerobic activities of all followed enzymes.

The studies with *E. coli* showed that paraquat induces about 40 proteins in this bacterium, most of which have not been identified, including antioxidant and repair enzymes. Some of these are positively or negatively regulated at the transcriptional level (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990). The Mn-SOD gene (*sod*A) itself is regulated by at least six global control systems, which coordinate optimal Mn-SOD induction under aerobic or anaerobic conditions (Hassan and Schrum, 1994). The TBARS content, which is known as biomarker of lipidic peroxidation found in our experiment at the presence of 0.5 mM and 1.0 mM paraquat was similar under the aerobic or anaerobic conditions. This reveals that even increased aerobic SOD and GSHPx were not able to prevent the oxidative damage. On the other hand, the inhibition of these enzymes at 2.0 mM paraquat resulted in far more damage, especially under the aerobic conditions when the TBARS values increased about four times. Our results indicate the complexity of *S. bovis* to respond efficiently to the oxyradical damage. There are probably other redox sensitive proteins which in collaboration with antioxidant enzymes could ensure the survival of this bacterium under the oxidative stress.

REFERENCES

Bannister J.V., Bannister W.H., Rotilio G. (1987): Aspects of the structure, function, and application of superoxide dismutase. CRC Crit. Rev. Biochem., *22*, 118–180.

Beauchamp C.O., Fridovich I. (1971): Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem., *44*, 276–287.

Belov L.T., Fridovich I. (1994): *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. J. Biol. Chem., *269*, 25310–25314.

Beyer W., Imlay J., Fridovich I. (1991): Superoxide dismutases. Prog. Nucleic Acid Res., *40*, 221–253.

Bradford M.M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72,* 248–254.

Britton L., Malinowski D.P., Fridovich I. (1978): Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. J. Bacteriol., 134, 229–236.

Bryant M.P. (1972): Commentary on the Hungate techniques for culture of anaerobic bacteria. Am. J. Clin. Nutr., *25*, 1324–1328.

Chang S.K., Hassan H.M. (1997): Characterization of superoxide dismutase in *Streptococcus thermophilus*. Appl. Environ. Microbiol., *63*, 3732–3735.

Flohé L., Günzler W.A. (1984): Assays of glutathione peroxidase. Method. Enzymol., *105,* 114–121.

Flohé L., Ötting F. (1984): Superoxide dismutase assays. Method. Enzymol., *105*, 93–104.

Fridovich I. (1978): The biology of oxygen radicals. Science, *201*, 875–880.

Fridovich I. (1997): Superoxide anion radical (O_2^-), superoxide dismutases, and related matters. J. Biol. Chem.., *272*, 18515–18517.

Fulghum R.S., Worthington J.M. (1984): Superoxide dismutase in ruminal bacteria. Appl. Environ. Microbiol., *48*, 675–677.

Gregory E.M., Dapper C.H. (1983): Isolation of iron-containing superoxide dismutase from *Bacteroides fragilis*: reconstitution as a Mn-containing enzyme. Arch. Biochem. Biophys., *220*, 293–300.

Gregory E.M., Fridovich I. (1973): The induction of superoxide dismutase by molecular oxygen. J. Bacteriol., *114,* 543–548.

Greenberg J.T., Monach P., Chou J.H., Josephy P.D., Demple B. (1990): Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci USA, *87,* 6181–6185.

Gutteridge J.M.C. (1984): Ferrous ion-EDTA-stimulated phospholipid peroxidation. Biochem. J., *224,* 697–701.

Hassan H.M. (1989): Microbial superoxide dismutases. Adv. Genet., *26,* 65–97.

Hassan H.M., Fridovich I. (1977): Regulation of the synthesis of superoxidedismutase in *Escherichia coli*: induction by methyl viologen. J. Biol. Chem., *252,* 7667–7672.

Hassan H.M., Fridovich I. (1978): Regulation of the synthesis of catalase and peroxidase in *Escherichia coli.* J. Biol. Chem., *253,* 6445–6450.

Hassan H.M., Fridovich I. (1979): Paraquat and *Escherichia coli*: mechanism of production of extracellular superoxide radical. J. Biol. Chem., *254,* 10846–10852.

Hassan H.M., Schrum L.W. (1994): Roles of manganese and iron in the regulation of the biosynthesis of manganese-superoxide dismutase in *Escherichia coli*. FEMS Microbiol. Rev., *14,* 315–324.

Kirby T., Blum J., Kahane I., Fridovich I. (1980): Distinguishing between Mn-containing and Fe-containing superoxide dismutases in crude extracts of cells. Arch. Biochem. Biophys., *201*, 551–555.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, *227,* 680–685.

Mead L.J., Jones G.A. (1981): Isolation and presumptive identification of adherent epithelial bacteria ("Epimural bacteria") from the ovine rumen wall. Appl. Environ.. Microbiol., *41,* 1020–1028.

Meier B., Barra D., Bossa F., Calabrese L., Rotoli G. (1982): Synthesis of either Fe- or Mn-superoxide dismutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied. J. Biol. Chem., *257,* 13977–13980.

Ose D.E., Fridovich I. (1976a): Superoxide dismutase: reversible removal of manganase and its substitution by cobalt, nicel, or zinc. J. Biol. Chem., *251,* 1217–1218.

Ose D.E, Fridovich I. (1976b): Manganese-containing superoxide dismutase from *Escherichia coli:* reversible resolution and metal replacement. Arch. Biochem. Biophys., *194,* 360–364.

Pinto M.C., Mata A.M., López-Barea J. (1984): Reversible inactivation of *Saccharomyces cerevisiae* glutathione reductase under reducing conditions. Arch. Biochem. Biophys., *228,* 1–12.

Steinman H.M., Ely B. (1990): Copper-zinc superoxide dismutase of *Caulobacter crescentus*: cloning, sequencing, and mapping of the gene and periplasmic location of the enzyme. J. Bacteriol., *172,* 2901–2910.

Storz G., Tartaglia L.A., Farr S.B., Ames B.N. (1990): Bacterial defenses against oxidative stress. Trends Genet., *6,* 363–368.

Štyriak I., Španová A., Montagová H., Kmeť V. (1994): Isolation and characterization of a new ruminal bacteriophage lytic to *Streptococcus bovisv*. Curr. Microbiol., *28,* 355–358.

Tsaneva I.R., Weiss B. (1990): SoxR, a locus governing a superoxide response regulon in *Escherichia coli* K-12. J. Bacteriol., *172,* 4197–4205.

Whitehead T.R., Cotta M.A. (2000): Development of molecular methods for identification of *Streptococcus bovis* from human and ruminal origin. FEMS Microbiology, *182,* 237–240.

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