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Oxygen Toxicity in *Streptococcus mutans*: Manganese, Iron, and Superoxide Dismutase

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When cultured anaerobically in a chemically defined medium that was treated with Chelex-100 to lower its trace metal content, *Streptococcus mutans* OMZ176 had no apparent requirement for manganese or iron. Manganese or iron was necessary for aerobic cultivation in deep static cultures. During continuous aerobic cultivation in a stirred chemostat, iron did not support the growth rate achieved with manganese. Since the dissolved oxygen level in the chemostat cultures was higher than the final level in the static cultures, manganese may be required for growth at elevated oxygen levels. In medium supplemented with manganese, cells grown anaerobically contained a low level of superoxide dismutase (SOD) activity; aerobic cultivation increased SOD activity at least threefold. In iron-supplemented medium, cells grown anaerobically also had low SOD activity; aerobic incubation resulted in little increase in SOD activity. Polyacrylamide gel electrophoresis of the cell extracts revealed a major band and a minor band of SOD activity in the cells grown with manganese; however, cells grown with iron contained a single band of SOD activity with an R_f value similar to that of the major band found in cells grown with manganese. None of the SOD activity bands were abolished by the inclusion of 2 mM hydrogen peroxide in the SOD activity strain. *S. mutans* may not produce a separate iron-containing SOD but may insert either iron or manganese into an apo-SOD protein. Alternatively, iron may function in another activity (not SOD) that augments the defense against oxygen toxicity at low SOD levels.

Streptococcus mutans, a member of the human oral flora, plays an important role in the development of dental caries (16). After the bacteria adhere to certain components of saliva that have adsorbed to tooth surfaces, the disease is initiated by microbial production of acid that subsequently dissolves tooth enamel (19, 23). Of importance to present studies are reports that elevated concentrations of manganese in drinking water or tooth enamel may promote the incidence of caries (1, 4, 8, 13). The effects of several trace metals on growth during continuous cultivation of *S. mutans* have been studied. *S. mutans* BHT requires manganese for growth (26), and in manganese-supplemented medium, steady-state growth of *S. mutans* OMZ176 is stimulated by iron, zinc, cobalt, or tin (2). Both strains also require magnesium (2, 26).

One of the important functions of manganese in biological systems is to serve as a cofactor for superoxide dismutase (SOD), an enzyme essential in the defense against oxygen toxicity (11). Procaryotes synthesize both manganese-containing SOD (MnSOD) and iron-containing SOD (FeSOD) (11), and *S. mutans* is known to produce MnSOD (28). Although *S. mutans* contains only an anaerobic type of energy-generating system in which carbohydrates are fermented to yield primarily lactic acid, the microorganism is aerotolerant and can be cultivated in the presence of oxygen (7). In spite of the lack of the major enzymes of oxygen metabolism (including respiratory cytochromes and catalase), various strains of *S. mutans* take up oxygen and (depending on the strain) may release hydrogen peroxide, superoxide, or both (27).

In the present study, no requirement for manganese or iron was apparent during anaerobic cultivation of *S. mutans* OMZ176; however, supplementation with manganese or iron was necessary for aerobic growth, and manganese was essential at elevated oxygen concentrations. Evidence was obtained that *S. mutans* may employ either manganese or iron as a cofactor for SOD, perhaps utilizing the same protein moiety. These data may explain the cariogenic potential assigned to manganese.

MATERIALS AND METHODS

Bacterial strain, culture medium, and culture procedures. *S. mutans* OMZ176 and procedures for maintenance of cultures have been described (2). The chemically defined medium (modified FMC medium) and the method for treatment of the medium with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) to lower trace metal contamination were reported previously (2). The sterile medium was supplemented before use with filter-sterilized solutions of high-purity (Johnson-Matthey, & Co., Inc., New York) $MgSO_4 \cdot 7H_2O$ (420 μM magnesium) and the desired levels of $MnSO_4 \cdot 5H_2O$ and $FeSO_4 \cdot 7H_2O$. The dissolved O_2 concentration in the medium was measured with a dissolved-O_2 probe (model 97-08-00; Orion Industries, Ltd., Chicago, Ill.) connected to a Radiometer model 26 meter and with dissolved-O_2 test kits obtained from Chemetrics, Calverton, Va.; during cultivation in the chemostat, the level of dissolved O_2 was continuously monitored with a dissolved-O_2 analyzer (model DO-50; New Brunswick Scientific Co., Inc., Edison, N.J.) equipped with a sterilizable probe (New Brunswick model M 1016-2808, without stainless-steel holder). Incubation was at 35°C. The chemostat, constructed of Teflon, has been described (26), and procedures used for continuous cultivation of *S. mutans* OMZ176 in the chemostat at a constant dissolved-O_2 concentration of 0.4 ppm (μl/liter), with continuous input of

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CO_2, were identical to those reported earlier (2). Inocula for the chemostat cultures were grown anaerobically in medium containing manganese (180 μM) and iron (18 μM). Before inoculation of medium in the chemostat, the cells were washed by centrifugation with medium lacking the trace metal supplements. All other cultures were not stirred (static). For anaerobic cultivation in an atmosphere of 90% N_2–5% H_2–5% CO_2, a modified Forma Scientific, Inc. (Marietta, Ohio) Anaerobic System model 1024 (with an add-on unit to increase work space and special inlet ports for vacuum lines and cables for pH and other types of probes) was used. The entire anaerobic chamber was warmed to 35°C with a heater-circulator (Coy Laboratories) and an auxiliary fan; metal portions of the unit were covered externally with 2-in. (5.08-cm) insulation to retard heat loss. Before inoculation, the sterile medium was equilibrated with the anaerobic atmosphere by stirring for 48 h or by sparging the stirred medium for 24 h, using an aquarium pump and an in-line filter (pore diameter, 0.22 μm) to maintain sterility. For aerobic cultivation in air, the carbonate requirement of the organism was satisfied by supplementing the medium with high-purity 7 mM potassium carbonate (Johnson-Matthey). For aerobic dose-response assays to determine the effects of manganese and iron on growth, the carbonate requirement was satisfied by incubation in an atmosphere of 95% air–5% CO_2 with medium lacking the potassium carbonate supplement. The dose-response assays were done in tubes (16 by 150 mm) containing 10 ml of medium.

Preparation of cell extracts; assays for SOD activity. Inocula for cultures to be used for preparation of cell extracts were obtained from the anaerobically grown, fourth successive transfer of cells in medium lacking the manganese or iron supplements. Usually, 1-liter amounts of medium (in 2.8-liter Fernbach flasks), supplemented with manganese (180 μM) and iron (18 μM) or without one or both of these metal supplements, were inoculated. These cultures were incubated aerobically or anaerobically. At near maximal stationary phase, cells were harvested by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.8), and finally suspended in 20 ml of this buffer. Extracts were prepared from these cells by rapid agitation with 0.5-mm glass beads (Bead Beater; Biospec Products, Bartlesville, Okla.), using 2-min treatments for a total time of 10 min with 10 min of cooling in ice between treatments. The disrupted cells were centrifuged at 30,000 × *g* at 4°C for 20 min, and the supernatant was dialyzed for 18 to 24 h at 4°C with 6 liters of the 50 mM potassium phosphate buffer. Protein in the extracts was determined by the method of Lowry et al. (20). To minimize exposure of anaerobically grown cells to oxygen, centrifugation was done in vessels that were sealed before removal from the anaerobic chamber and that were returned to the chamber before opening. Cell disruption was performed in the anaerobic chamber with phosphate buffer that was equilibrated with the anaerobic atmosphere.

Spectrophotometric measurements of SOD activity in cell extracts were made by the method of McCord and Fridovich (21) with a recording spectrophotometer (GCA McPherson). One unit of SOD activity was defined as 50% inhibition of the rate of cytochrome *c* reduction in this assay. Extracts also were fractionated by electrophoresis in 7.5 or 10% polyacrylamide gels (10-cm length) with 50 to 150 μg of protein and 2 mA per gel tube (model CWS-300; Savant Instruments, Inc., Hicksville, N.Y.). After electrophoresis, the SOD activity was located in the gels by photochemical staining (3, 5). Sensitivity of the SOD to H_2O_2 was determined by the addition of 2 mM H_2O_2 to the staining solution (5, 6).

EDDA inhibition of growth and reversal of growth inhibition. To study the effects of the metal-chelating agent ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA; Sigma Chemical Co., St. Louis, Mo.) on growth of *S. mutans* OMZ176, we modified the disk diffusion method described by Ong et al. (24) as follows. Molten Todd-Hewitt agar (containing 1 mg of EDDA per ml) at 50°C was seeded with 10^5 CFU/ml, immediately poured into plates, and allowed to solidify. Paper disks (0.25 in. [ca. 6.3 mm]; Difco Laboratories, Detroit, Mich.) were treated before use by soaking in 0.025 M EDTA and then washing with sufficient high-purity water (prepared by passage through a reverse osmosis-charcoal deionizer column system [Millipore Corp., Bedford, Mass.]) to remove the EDTA. They were sterilized by autoclaving. The desired volumes of the filter-sterilized test solutions were pipetted onto the sterile disks, which then were aseptically placed on the surface of the seeded agar. Agar plates were incubated anaerobically or aerobically (95% air–5% CO_2) at 35°C; zones of growth around the disks at 24 to 48 h indicated reversal of EDDA inhibition of growth by the test solution. The EDDA was deferrated before use (25).

RESULTS

Manganese requirement for continuous cultivation in the chemostat. The Teflon chemostat (26) was used to examine the manganese requirement of *S. mutans* OMZ176. The cells were first grown as a batch culture in the chemostat (without input of fresh medium) until growth reached near maximum stationary phase, and then the input of medium was started. A dissolved-O_2 concentration of 0.4 ppm (μl/liter) was maintained. When medium containing manganese and iron was used, apparent steady-state growth was reached by 22 h after input of medium was started (Fig. 1A). If the medium lacked the manganese supplement, there was no difference in growth from the manganese-supplemented culture during the initial batch phase of cultivation. However, a detectable steady-state growth was not established at the dilution rate used, and at 26 h after medium input was initiated, the population had fallen below the limits detectable by dry weight or turbidity measurements (Fig. 1B). The addition of manganese (18 μM) to this culture and to the incoming medium allowed the culture to establish a steady-state growth level that was similar to that noted in cultures containing 180 μM manganese (Fig. 1). A subsequent increase in the manganese concentration to 36 μM did not increase the level of equilibrium growth. These data show that manganese was essential for achieving a detectable steady state with the conditions and dilution rate employed. The initial growth in the absence of manganese as a batch culture (Fig. 1B) probably was due to the carry-over of manganese with the inoculum. This possibility is supported by the observation that, in aerobic batch cultures, inocula previously cultured in manganese-containing medium were able to achieve maximal growth in the absence of manganese, but the resulting manganese-starved cells failed to grow when subsequently transferred to medium without manganese.

Manganese reversal of EDDA inhibition of growth. The chelating agent EDDA inhibits growth of certain bacteria. This inhibition often can be reversed by the addition of iron or a siderophore (microbial iron transport cofactor) (e.g., reference 24). We found that *S. mutans* OMZ176 was unable

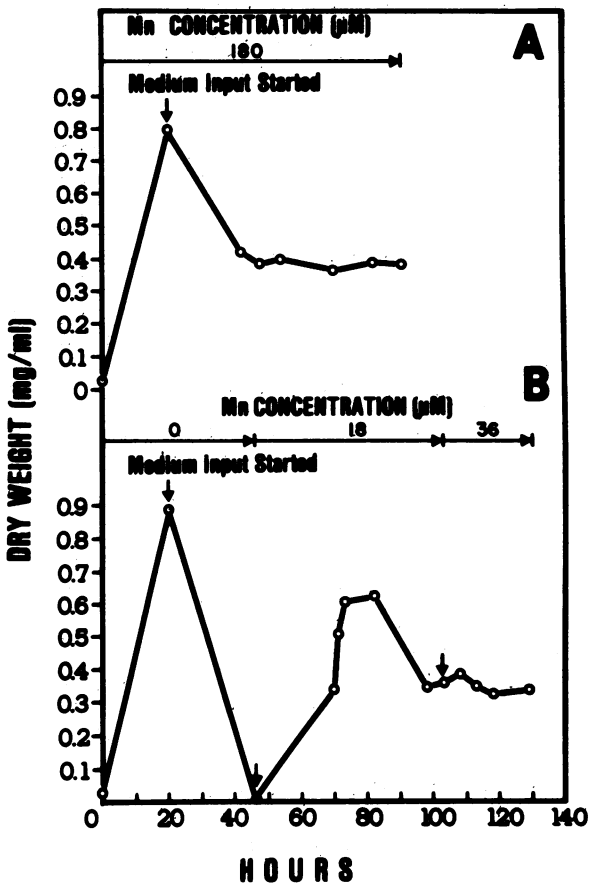

FIG. 1. Effect of manganese on aerobic continuous growth of *S. mutans* OMZ176 in the Teflon chemostat. Cultures first were grown as batch cultures in medium supplemented with 180 μM manganese (A) or no manganese (B), and at 20 h medium (at the respective manganese concentrations) input was started at a dilution rate of 0.13/h. In the culture (B) that was initially grown in medium without manganese, the manganese concentration was raised to 18 μM at 46 h and 36 μM at 103 h. In both cultures, the medium also contained 420 μM magnesium and 18 μM iron.

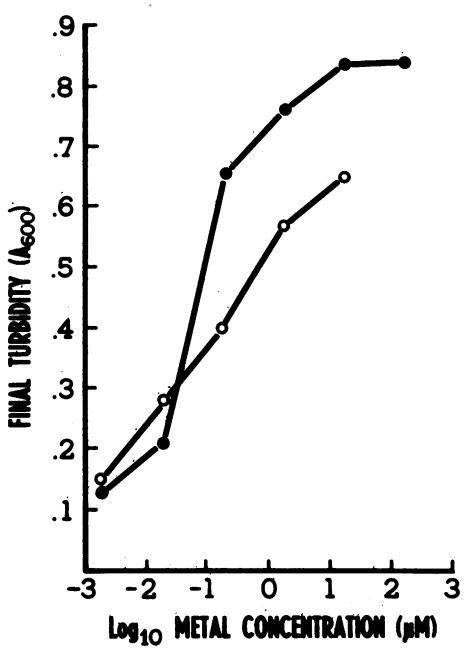

FIG. 2. Effect of manganese and iron on aerobic growth of *S. mutans* OMZ176. Cultures in unshaken tubes were incubated at 35°C in 95% air–5% CO_2 in medium (supplemented with 420 μM magnesium) containing either manganese (●) or iron (○). Inocula were grown anaerobically in medium without manganese or iron.

to grow aerobically on Todd-Hewitt agar containing 1 mg of EDDA per ml, but no inhibition of growth was apparent on plates incubated anaerobically. Growth inhibition on aerobically incubated plates was reversed by disks containing manganese (0.02 μmol), lactic acid (3.8 μmol), or acetic acid (13.3 μmol). Iron (at concentrations up to 0.54 μmol per disk) did not reverse growth inhibition; calcium (1.5 μmol), magnesium (5 μmol), and strontium (0.15 μmol) also were ineffective. These data indicate that chelation of manganese by EDDA may be the primary cause of EDDA inhibition of *S. mutans* OMZ176. Manganese is chelated by EDDA only above pH 6 (10, 18), and this may explain the reversal of growth inhibition by acidic conditions. Several siderophores (including enterobactin, agrobactin, and ferrioxamine B) failed to reverse EDDA inhibition.

Manganese requirement for aerobic (but not anaerobic) growth; replacement by iron in static cultures. *S. mutans* OMZ176 could be grown anaerobically for multiple transfers (at least 20) in medium without added iron and manganese. There was no apparent stimulation of growth upon the addition of either manganese, iron, or both (at concentrations up to 180 μM and 18 μM, respectively) to anaerobically incubated cultures inoculated with cells obtained from the tenth transfer in medium lacking manganese or iron (data not shown). Cells cultured anaerobically in the absence of both manganese and iron were used to inoculate tube cultures that were then incubated aerobically. Only slight growth occurred without a manganese supplement; the optimal concentration of manganese was about 18 μM (Fig. 2), a value similar to that found to be effective in the chemostat. Iron could partly replace the manganese requirement for aerobic growth in these static cultures, although the final population densities achieved in cultures supplemented only with iron were consistently less than those achieved in cultures containing at least 18 μM manganese (Fig. 2). The final dissolved-O_2 level in these cultures was 0.1 ppm (μl/liter) or less.

SOD activity. Since procaryotic cells utilize manganese and iron as cofactors for SOD enzymes (11), we measured SOD activity in cell extracts prepared from cells grown under various conditions (Table 1). Spectrophotometric assays of extracts of cells grown anaerobically without manganese or iron showed no detectable SOD activity; however, extracts of cells grown anaerobically with manganese, iron, or both metals contained low SOD activity. Aerobic growth with manganese or with both manganese and iron increased SOD activity in the extracts at least threefold. The SOD level in cells grown aerobically with iron only was slightly higher (probably insignificant) than that in cells grown anaerobically with iron. If the extracts were heated for 10 min in a boiling water bath, all SOD activity was destroyed, suggesting an enzymatic reaction.

Gel electrophoresis: effect of H_2O_2 on SOD activity. Polyacrylamide gel electrophoresis (with subsequent photochemi-

TABLE 1. Effect of growth with manganese and iron on SOD activity in extracts of *S. mutans* strain OMZ176

Metal(s) added[a]	Activity (U/mg of protein) under culture conditions:	
	Anaerobic	Aerobic
None	0	NG[b]
Manganese	6	22
Iron	2	5
Manganese, iron	4	22

[a] Where indicated, manganese was added at 180 μM; iron was added at 18 μM.
[b] NG, No growth aerobically without manganese or iron.

cal staining of the gels to visualize SOD) of extracts prepared from cells grown aerobically with manganese revealed a major band and a minor band of SOD activity (Fig. 3). Gels prepared using extracts of cells grown aerobically with only iron contained a single band of SOD that appeared to migrate to the same position as the major band noted in gels prepared from cells grown with manganese (Fig. 3). Similar migration patterns were observed in 7.5 and 10% acrylamide gels. The major SOD activity in *S. mutans* is known to be MnSOD (28), and it is likely that in present studies cells grown with manganese produced MnSOD. However, manganese- and iron-deficient cells (that had been repeatedly transferred in anaerobic medium without manganese or iron) were used as inocula for the aerobic cultures, and it is possible that, in medium supplemented only with iron, the manganese-starved cells synthesized FeSOD. Since sensitivity to H_2O_2 is a characteristic of FeSOD (5, 12), we determined the effect of this inhibitor on SOD activity in *S. mutans*. The SOD activity was not abolished in any of the bands by the inclusion of 2 mM H_2O_2 in the activity stain (Fig. 3). H_2O_2 caused some loss of the staining intensity of all the bands, but this effect has been noted previously (5). To assure that inhibition of a known FeSOD would have been evident in our assays, we prepared gels with extracts of *Escherichia coli* B and noted that the activity of FeSOD was not visualized in gels stained in the presence of 2 mM H_2O_2.

DISCUSSION

Anaerobic growth of *S. mutans* OMZ176 did not require manganese or iron supplements. If iron or manganese is necessary for anaerobic cultivation of *S. mutans*, then the levels required by this strain must be low. Aerobic growth of the organism required manganese at a fairly high level (18 μM) for continuous cultivation in a chemostat and for static cultivation in tube cultures. In the static tube cultures, iron replaced the manganese requirement, but at the same concentration in continuous cultures, iron could not support the growth rate achieved with manganese. Since the final dissolved oxygen level in the static cultures was less than the level in the chemostat cultures, iron may have permitted growth at only very low oxygen levels, whereas manganese was needed for growth at higher oxygen concentrations. This conclusion may be supported by experiments with the chelating agent EDDA. This chelator inhibited aerobic, but not anaerobic, growth of *S. mutans* on plates of agar; inhibition was reversed by manganese but not by iron, suggesting that chelation of manganese by EDDA rendered the metal unavailable to the organism.

Aerobic cultivation with manganese increased SOD activity at least threefold, showing that the response of *S. mutans* to oxygen stress was similar to that of other bacteria, including *Streptococcus faecalis* and *Streptococcus sanguis* (6, 9, 15, 17). *S. mutans* grown only with iron had low SOD activity, and electrophoresis of cell extracts revealed a single band of activity that appeared to migrate to the same position as the major band of SOD demonstrated in cells grown with manganese. All of the SOD bands could be visualized when stained in the presence of 2 mM H_2O_2. Since sensitivity to H_2O_2 is a characteristic of many FeSOD enzymes (12), this finding may argue that FeSOD was not present in these extracts. However, the culture procedures used (in which manganese-deficient cells were grown aerobically only with iron) suggest that, in the absence of manganese, *S. mutans* utilized iron as a cofactor for SOD.

The data may indicate at least two possibilities. *S. mutans* may not produce a separate FeSOD. The organism may insert either iron or manganese into an apo-SOD that is capable of accepting either metal but which has preference for manganese as a cofactor. Some support for this suggestion may be found in the recent report that *Propionibacterium shermanii* produces, depending on the metal available in the culture medium, either FeSOD or MnSOD and utilizes a single protein moiety for this activity (22). *Bacteroides fragilis* also synthesizes SOD that accepts either manganese or iron (14). Alternatively, it is possible that traces of manganese remaining in the medium permitted synthesis of

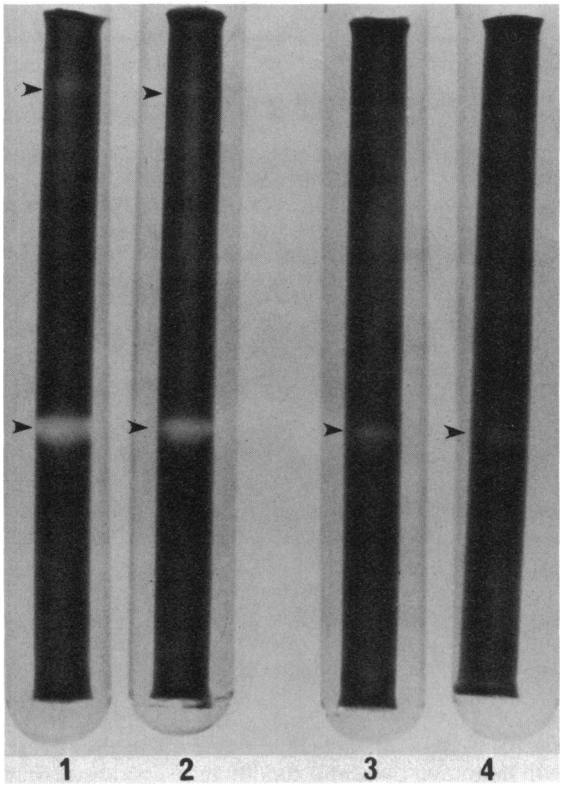

FIG. 3. Polyacrylamide gel electrophoresis of cell extracts showing SOD activity demonstrated by photochemical staining. Arrows indicate positions of SOD bands. Gels were loaded (100 μg of protein) with extracts of cells cultured aerobically with (1) manganese, (2) manganese with H_2O_2 added to activity stain, (3) iron, and (4) iron with H_2O_2 added to activity stain. When added, manganese was present at 180 μM, iron was present at 18 μM, and H_2O_2 was present at 2 mM.

some MnSOD in the absence of added manganese. However, this low level of activity may have been inadequate for aerobic growth. Iron may have participated in another function (not SOD) that augmented the low MnSOD activity, thereby permitting growth at low oxygen levels without the addition of manganese. This suggestion may explain the report (2) that the addition of iron to continuous cultures growing in manganese-containing medium increases the level of steady-state growth. To resolve these possibilities, it will be necessary to determine the metal present in SOD purified from cells grown aerobically only with iron.

Present data also may explain the known cariogenic potential of manganese. Evidence cited above indicates a direct association between manganese concentrations in the oral cavity and the incidence of dental caries. Manganese will be required for *S. mutans* to move from anaerobic niches to regions of elevated oxygen tension in the oral cavity. Increased availability of manganese should permit extensive proliferation of the organism and thereby increase dental caries. Development of means to limit manganese uptake by *S. mutans* may assist in the control of this disease.

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