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Manganese Oxidation by *Leptothrix discophora*

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Cells of *Leptothrix discophora* SS1 released Mn^{2+}-oxidizing factors into the medium during growth in batch culture. Manganese was optimally oxidized when the medium was buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) at pH 7.5. Manganese-oxidizing activity in the culture medium in which this strain had been grown previously was sensitive to heat, phosphate, Tris, NaN_3, $HgCl_2$ NaCl, sodium dodecyl sulfate, and pronase; 0.5 mol of O_2 was consumed per mol of MnO_2 formed. During Mn^{2+} oxidation, protons were liberated. With sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two protein-containing bands were detected in the spent culture medium. One band had an apparent molecular weight of 110,000 and was predominant in Mn^{2+}-oxidizing activity. The second product (M_r 85,000) was only detected in some cases and probably represents a proteolytic breakdown moiety of the 110,000-M_r protein. The Mn^{2+}-oxidizing factors were associated with the MnO_2 aggregates that had been formed in spent culture medium. After solubilization of this MnO_2 with ascorbate, Mn^{2+}-oxidizing activity could be recovered.

Although manganese-oxidizing bacteria have been recognized since the beginning of this century (4), many aspects of the mechanism of manganese oxidation have remained obscure. In many cases it is still unclear whether manganese-oxidizing microorganisms gain an advantage from the process (3, 11, 19, 22). Although it is assumed that some organisms produce specific macromolecules that catalyze the oxidation process (so-called direct catalysis; see reference 19), the data supporting this assumption are usually based on experiments with crude cell extracts and studies of the effects of inhibitors on manganese oxidation (7, 8, 10, 12)

Bacterial species belonging to the genus *Leptothrix* oxidize manganese (6, 22). Manganese oxide deposits are never found inside cells, but always in association with extracellular polymers (13). In the species *Leptothrix discophora*, these polymers either occur in structured sheaths (18, 22), are randomly oriented (1), or occur freely in the medium (13, 18). *L. discophora* SS1 lost its ability to produce a structured sheath shortly after its isolation (1). It continued to produce extracellular polymers (1, 13) and retained the ability to oxidize manganese (1). In this study we show that not only cells but also macromolecules present in the spent culture medium are able to catalyze the oxidation of manganese. The nature of these manganese-oxidizing macromolecules was investigated by partial characterization of their activity in the spent culture medium and by their identification in sodium dodecyl sulfate (SDS)-polyacrylamide gels.

MATERIALS AND METHODS

Organism. *L. discophora* SS1 was kindly provided by W. C. Ghiorse (Cornell University, Ithaca, N.Y.). Electron microscopic examinations revealed that this strain did not form well-defined sheaths (data not shown), as shown by Adams and Ghiorse (1, 2).

Growth conditions. The bacteria were grown at room temperature in batch cultures (800 ml) in 1-liter flasks with a continuous supply of sterile air. The cultures were stirred continuously. The medium contained (per liter of deionized water): 0.5 g of yeast extract (Difco Laboratories), 0.5 g of Casamino Acids (Difco), 5 mM D(+)-glucose, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.5, 0.48 mM $CaCl_2$, 0.83 mM $MgSO_4$, 3.7 μM $FeCl_3$, and 1 ml of trace element solution. The trace element solution contained (per liter of deionized water): 10 mg of $CuSO_4 \cdot 5H_2O$, 44 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of $CoCl_2 \cdot 6H_2O$, and 13 mg of $Na_2MoO_4 \cdot 2H_2O$. Prior to the addition of HEPES, glucose, and the Casamino Acids, the medium was autoclaved for 20 min at 120°C. Then HEPES, glucose (both 0.2-μm filter sterilized), and Casamino Acids (sterilized for 40 min at 110°C) were added as concentrated solutions. *L. discophora* SS1 was inoculated on solid agar plates containing the same nutrients plus 0.1 mM $MnCl_2$ (filter sterilized). During growth, the pH of the culture dropped from 7.5 to 7.2. Cells were harvested by centrifugation (15 min, 10,000 × g, 4°C), washed once, and suspended in 10 mM HEPES (pH 7). Suspensions were kept at 4°C prior to use.

Ultrafiltration. Batch cultures were centrifuged, and spent culture medium was concentrated under pressure (2.5 atm [ca. 250 kPa]) over filters with molecular weight cutoffs of 10,000 (10K), 50K, 100K, and 300K (Amicon B.V., Oosterhout, The Netherlands).

Determination of MnO_2 concentration. The MnO_2 concentration was measured colorimetrically with the Leuco Berbelin blue assay (15). Samples (0.1 ml) were added to 0.5 ml of 0.04% Leuco Berbelin blue in 45 mM acetic acid, and the absorbance was measured at 620 nm. Any cells present in the samples were removed by centrifugation prior to measurement of the absorbance. The oxidation of Leuco Berbelin blue proceeded within a few seconds with the development of a blue color. Standard curves with $KMnO_4$ showed that the absorbance was linear up to A_{620} = 1.50.

Determination of oxygen consumption and proton production. O_2 consumption at 25°C was measured with a Clark oxygen electrode. Manganese oxidation was started by injecting 40 μl of 5 mM $MnCl_2$ into 2 ml of concentrated spent culture medium. In parallel experiments, MnO_2 formation was measured with the aid of the Leuco Berbelin blue assay.

Proton production was measured with a combined glass electrode (Schott-Gerate, Hofheim a. Ts., Federal Republic of Germany) connected to a pH meter (type CG805; Schott-Gerate), and the output of the pH meter was monitored with

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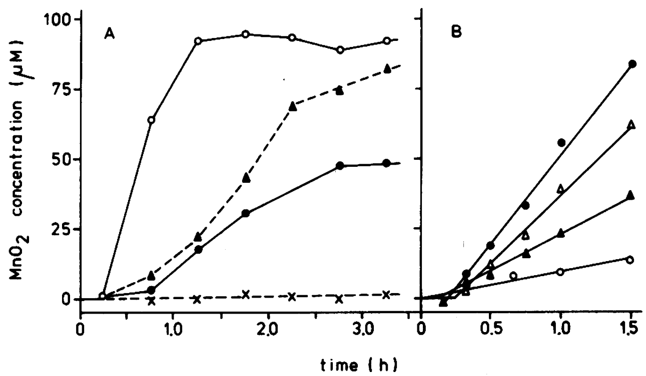

FIG. 1. (A) Manganese oxide formation by cells and spent culture medium of *L. discophora*. Symbols: ▲, cells were harvested from a batch culture (OD_{660} = 0.83), washed once with 10 mM HEPES, and suspended in 10 mM HEPES (pH 7) at an OD_{660} of 3.0; ●, spent culture medium of the batch culture; ○, residue (20 ml) after ultrafiltration of 500 ml of spent culture medium over a 10K filter; ×, filtrate after ultrafiltration. At 0 min 100 μM $MnCl_2$ was added in each case. (B) Manganese oxide formation by supernatant fractions obtained after successive incubations of a cell suspension with HEPES buffer. Cells from 50 ml of a batch culture were harvested and suspended in 5 ml of 10 mM HEPES (pH 7). After 30 min the suspension was centrifuged (10 min, 10,000 × *g*), and the supernatant fluid was tested for manganese oxide formation (●). The pellet was suspended in another 5 ml of HEPES, and the same procedure was repeated three times (△, ▲, and ○). The reaction was started by addition of 100 μM $MnCl_2$ at 0 min.

a recorder. The deflection was directly proportional to the amount of protons added within the range of pH change during actual measurement (7.5 to 7.4). The reaction vessel contained 4 ml of concentrated spent culture medium and 6 ml of distilled water. Manganese oxidation was started by addition of 0.1 ml of 10 mM $MnCl_2$. At regular time intervals 0.1-ml samples were taken for measurement of the MnO_2 concentration by the Leuco Berbelin blue assay.

Polyacrylamide gel electrophoresis. The spent culture medium was concentrated 50-fold over a 10K ultramembrane filter as described above. This concentrate was mixed with an equal volume of buffer containing 0.125 M Tris, pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples (50 μl) were transferred to sample wells of a 7 or 10% SDS-polyacrylamide gel slab and electrophoresed in the standard manner with the discontinuous solvent system of Laemmli (16). After electrophoresis the gel was cut longitudinally into three parts. One part was stained with Coomassie brillant blue, and another with silver (17). The third part of the gel was used to detect Mn^{2+}-oxidizing activity. The gel was washed for 60 min with deionized water, which was replaced every 15 min. Subsequent incubation of the gel in a solution of 100 μM $MnCl_2$ in 10 mM HEPES, pH 7.5, for 1 to 2 h showed the presence of Mn^{2+}-oxidizing macromolecules by the development of brown deposits of MnO_2.

Isolation of Mn^{2+}-oxidizing factors from MnO_2 precipitates. After the formation of 70 μmol of MnO_2 by the spent culture medium from a batch culture (700 ml), the MnO_2 was sedimented by centrifugation (30 min, 20,000 × *g*). The MnO_2 was collected in 1 ml of 10 mM HEPES. By addition of 7 ml of a solution containing 40 mM ascorbic acid and 10 mM HEPES (pH 7), the MnO_2 was instantaneously solubilized. This solution was concentrated to 0.5 ml by ultrafiltration. The concentrate was diluted to 7.5 ml with the ascorbic acid solution and concentrated again to 0.5 ml. This procedure was repeated five times to remove all the Mn^{2+} from the solution. Subsequently, the preparation was washed eight times by ultrafiltration with 10 mM HEPES (pH 7) to remove ascorbic acid. The final preparation was adjusted to 1 ml and stored at −20°C

RESULTS

Oxidation of Mn^{2+} by cells and spent culture medium of *L. discophora* SS1. Cells were grown in batch culture until they reached the stationary phase of growth, corresponding to an OD_{660} of about 0.8. The cells were harvested, washed, and suspended in 10 mM HEPES (pH 7). This resting-cell suspension was able to oxidize 100 μM Mn^{2+} to MnO_2 in 3 h, after a lag phase of about 15 min (Fig. 1A). Cells harvested in the early, mid-, and late exponential growth phase also had the ability to oxidize Mn^{2+}. Although the Mn^{2+}-oxidizing activity varied from batch to batch, the general picture that emerged from experiments with numerous batches was that activity was higher in the stationary than in the exponential phase of growth. Spent culture medium also had the ability to oxidize Mn^{2+} (Fig. 1A). In general, during the growth of a culture, the activity of the spent culture medium underwent a development similar to that of the corresponding cell suspension; the highest activities were found in the stationary phase. The sum of the activity of the spent culture medium and the cells equaled that of the original culture. In general, the spent culture medium contained most of the activity. This activity could be concentrated over a filter with a molecular weight cutoff of 10K (Fig. 1A); the filtrate contained no activity at all. Neither HEPES buffer (10 mM, pH 7) nor uninoculated sterilized medium per se was able to oxidize Mn^{2+}. Ultrafiltration of active spent culture medium over filters with molecular weight cutoffs of 50K and 100K showed that the activity was again substantially enhanced in the concentrated solutions, but was completely absent in the filtrates. Ultrafiltration over a 300K filter resulted in increased activity in the concentrated solution, but some activity was also found in the filtrate.

Cells harvested from a batch culture released Mn^{2+}-oxidizing activity when suspended in HEPES buffer, pH 7.0, and incubated at room temperature (Fig. 1B). Successive incubations of the cells in fresh HEPES buffer resulted each time in the release of activity into the buffer, but the oxidizing activity gradually decreased (Fig. 1B). A similar release of activity was obtained when cells were incubated in HEPES buffer at 4°C, indicating that this process did not require active cellular metabolism.

Effect of temperature on Mn^{2+} oxidation. The Mn^{2+}-oxidizing activity of the spent culture medium was sensitive to high temperatures. After a concentrated spent culture medium was heated for 5 min at 50 or 90°C, 70 and 100%, respectively, of the original activity was lost (data not shown). The activity was stable to incubation of spent culture medium for 150 min at room temperature, but incubation for 2 or 5 days at 8°C resulted in loss of activity of 50 and 80%, respectively. Spent culture medium could be stored at least for 1 month at −80 or −20°C without significant loss of activity.

Effect of pH and buffer on Mn^{2+} oxidation. The Mn^{2+}-oxidizing activity of HEPES-buffered spent culture medium was strongly pH dependent. In Fig. 2 the oxidation rate is plotted as a function of the pH of the spent culture medium. Manganese oxidation proceeded optimally at pH 7.5. The

choice of the buffering system was important in measuring Mn^{2+} oxidation. When spent culture medium was buffered with both 1 mM HEPES and 25 mM Tris or 1 mM HEPES and 10 mM potassium phosphate at pH 7.0, no activity was measured at all. The presence of Tris at concentrations as low as 1 mM resulted in a 65% decrease in activity.

Inhibitors of Mn^{2+} oxidation. Azide inhibited Mn^{2+} oxidation at concentrations usually needed to decrease the activity of redox enzymes (Table 1). The activity was also sensitive to pronase, a mixture of proteolytic enzymes, the extent of inhibition being dependent on its concentration and the incubation time. Manganese oxidation was substantially inhibited by SDS, NaCl, and $HgCl_2$.

Identification of Mn^{2+}-oxidizing factors in SDS-polyacrylamide gels. The spent culture medium (concentrated 50-fold) was submitted to electrophoresis by the method of Laemmli (16). To detect Mn^{2+}-oxidizing activity in the gel, the gel had to be washed thoroughly to reduce the concentrations of Tris and SDS (see above). Subsequent incubation of the gel in a solution of 100 μM $MnCl_2$ in 10 mM HEPES, pH 7.5, showed the presence of an Mn^{2+}-oxidizing factor by the development of a brown band of MnO_2 (Fig. 3A). The band with the oxidizing activity corresponded with a Coomassie blue- as well as silver-stained product with an apparent molecular weight of 110,000. The MnO_2 band was marked by indentations in the gel, and the MnO_2 was solubilized by incubation of the gel in a 0.1% NH_2OH solution for about 15 min. Restaining the gel with silver or Coomassie blue confirmed the identification of the 110K product as an Mn^{2+}-oxidizing factor (Fig. 3A, compare lanes d and f with lanes c and b, respectively). No variation in the apparent molecular weight of the Mn^{2+}-oxidizing factor was observed when this experiment was repeated with samples from the spent culture media of other batches or of continuous cultures. The Mn^{2+}-oxidizing factor could not be stained for polyanions (e.g., acidic polysaccharides) with Alcian blue (data not shown). In some cases a second Mn^{2+}-oxidizing product with an apparent molecular weight of 85K was present (Fig. 3B). Both the 110K and the 85K products could be stained with silver. The 110K product was always predominant in Mn^{2+}-oxidizing activity. To investigate whether Mn^{2+}-oxidizing fragments could be produced from the 110K molecule by proteolytic action, the concentrated spent culture medium was treated with trypsin. By the action of this enzyme a small amount of an Mn^{2+}-oxidizing product with an apparent molecular weight of 95K was liberated (Fig. 3C).

Association of Mn^{2+}-oxidizing factors with MnO_2 aggregates. When aggregates of MnO_2 formed from Mn^{2+} by active spent culture medium were removed by centrifuga-

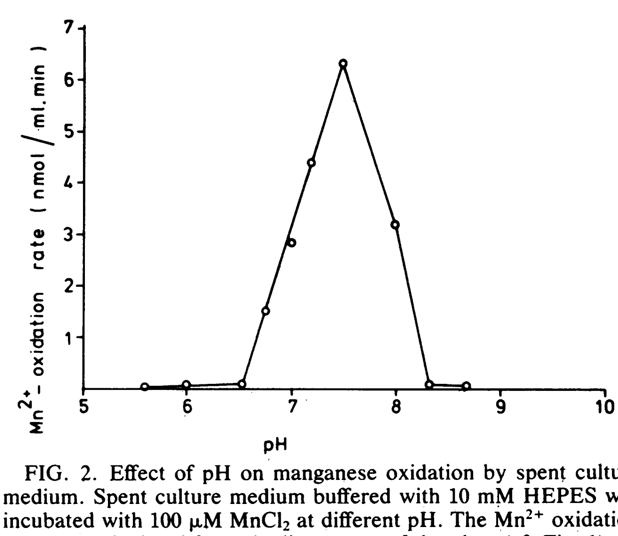

FIG. 2. Effect of pH on manganese oxidation by spent culture medium. Spent culture medium buffered with 10 mM HEPES was incubated with 100 μM $MnCl_2$ at different pH. The Mn^{2+} oxidation rate was calculated from the linear part of the plots (cf. Fig. 1).

TABLE 1. Inhibitors of manganese oxide formation by spent culture medium

Inhibitor	Concn	MnO_2 formation (nmol/ml per min)
NaN_3	0 mM	1.0
	0.1 mM	0.5
	1 mM	0.1
	10 mM	0
Pronase[a]	0 μg/ml	3.0
	5 μg/ml (45 min)	2.1
	5 μg/ml (90 min)	1.8
	50 μg/ml (15 min)	1.3
	50 μg/ml (45 min)	0.9
	500 μg/ml (30 min)	0.3
SDS	0%	1.3
	0.02%	1.2
	0.1%	0.5
NaCl	0 M	5.6
	0.1 M	2.6
	0.5 M	1.3
	1 M	0.6
$HgCl_2$	0 μM	4.9
	5 μM	3.6
	10 μM	3.2
	50 μM	0.6

[a] The time of incubation with pronase before addition of manganese is indicated in parentheses.

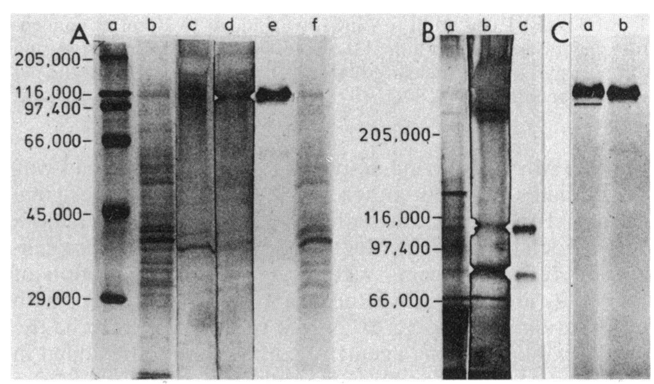

FIG. 3. SDS-polyacrylamide gel electrophoresis of concentrated spent culture medium. (A) Electrophoresis on a 10% gel. Staining was performed with Coomassie brillant blue (lane b) and silver (lane c). Manganese-oxidizing activity was detected by the formation of MnO_2 bands after incubation of the gel in an $MnCl_2$ solution (lane e). The MnO_2 band was marked with indentations. The MnO_2 was solubilized with hydroxylamine, and the gel was restained with silver (lane d) or Coomassie brillant blue (lane f). Molecular weights were calibrated with molecular weight protein standards (lane a). (B) Electrophoresis of spent culture medium from a different batch of cells on a 7% gel. The gel was stained with silver (lane a) or Mn^{2+} (lane c) or poststained with silver after dissolution of the MnO_2 bands as described above (lane b). (C) Concentrated spent culture medium was incubated with (lane a) trypsin (50 μg/ml) at room temperature for 2 h prior to electrophoresis on a 10% gel or (lane b) without trypsin. The gel was stained for Mn^{2+}-oxidizing activity as described above.

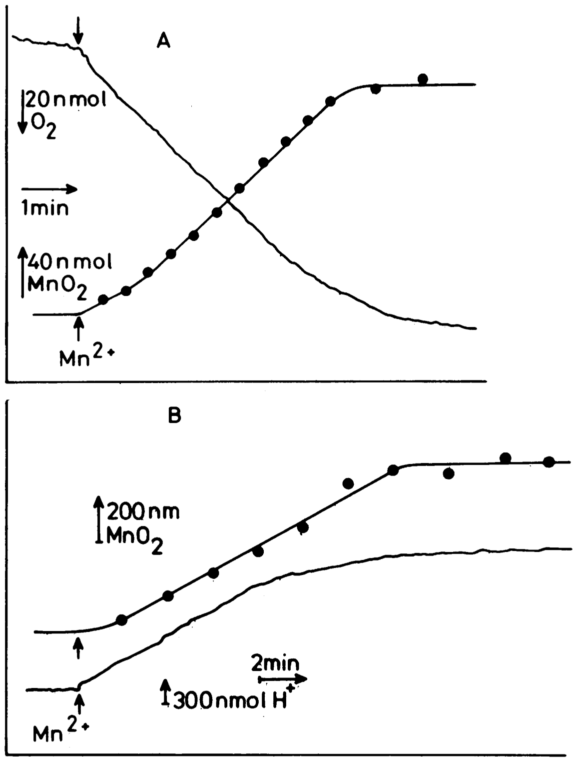

FIG. 4. Manganese oxide formation, oxygen consumption, and proton production by spent culture medium. (A) 200 nmol of $MnCl_2$ was added to 2 ml of concentrated spent culture medium at the point indicated by the arrow, and MnO_2 formation was measured (●). In a parallel experiment 200 nmol of $MnCl_2$ was added to another 2-ml sample of the same spent culture medium at the moment indicated by the arrow, and the O_2 consumption was recorded (—). The rate of O_2 consumption was corrected for the drift of the oxygen electrode. (B) 1,000 nmol of $MnCl_2$ was added to 10 ml of concentrated spent culture medium at the point indicated by the arrow, and MnO_2 formation was measured (●). In the same experiment, the pH was also recorded (—).

tion, the Mn^{2+}-oxidizing activity of the remaining fluid was substantially reduced, down to 0 to 10% of the original activity. To investigate whether the Mn^{2+}-oxidizing factors were associated with the MnO_2 aggregates, several complexing and reducing agents were tested for solubilization of the MnO_2 and release of the Mn^{2+}-oxidizing factors with preservation of activity. Ascorbate (pH 7) proved to be the most suitable reducing agent. Ascorbate had to be added in a fourfold excess over MnO_2 to reduce the latter and keep it reduced during the time necessary to wash Mn^{2+} out of the solution. Ascorbate itself was then replaced by 10 mM HEPES (pH 7.0). This final preparation (concentrated 700 times with respect to the original spent culture medium) oxidized Mn^{2+} at a rate of 16.6 nmol/ml per min. The oxidation rate of the original spent culture medium amounted to 0.7 nmol/ml per min. The 110K product was the main Mn^{2+}-oxidizing component in the concentrated preparation, as revealed by SDS gel electrophoresis. Staining with Coomassie blue or silver revealed the presence of several other proteins as well (data not shown).

Reaction sequence for Mn^{2+} oxidation. When concentrated spent culture medium was made anaerobic under nitrogen, no oxidation of Mn^{2+} took place. When aerobic conditions were reestablished, Mn^{2+} oxidation started immediately (data not shown). The rate of MnO_2 formation was about twice that of oxygen consumption, 19 and 9 nmol/ml per min, respectively (Fig. 4A). Calculation of the total amount of oxygen consumed during the oxidation of all available Mn^{2+} yielded a value of 51 nmol of O_2 per 100 nmol of MnO_2. In a second experiment, 48 nmol of O_2 was consumed during the formation of 100 nmol of MnO_2 (data not shown). These observations are in agreement with the following reaction equation: $Mn^{2+} + 0.5O_2 + H_2O \rightarrow MnO_2 + 2H^+$. According to this equation, one of the oxygen atoms of MnO_2 is derived from O_2 and the other one from H_2O, and protons are liberated during the production of MnO_2. We measured the pH change during Mn^{2+} oxidation in weakly buffered spent culture medium (Fig. 4B). MnO_2 was formed at a constant rate of 5 nmol/ml per min. In this experiment about 80% of the total Mn^{2+} was oxidized. During Mn^{2+} oxidation, protons were liberated. The initial rate of H^+ production was 15 nmol/ml per min. When about 45% of the total Mn^{2+} was oxidized, the rate of proton production decreased greatly. After cessation of the oxidation reaction, 212 nmol of H^+ had been produced per 100 nmol of MnO_2 formed. This experiment was repeated nine times, and it appeared that the ratio between the initial rates of proton and MnO_2 production was 3.8 ± 0.6 (mean $\pm$ standard deviation). The ratio of the final amounts of H^+ and MnO_2 formed was calculated to be 2.2 ± 0.3 after correction for the decrease in buffer capacity due to MnO_2 formation.

Azide inhibited the formation of MnO_2 by spent culture medium (Table 1). It had a similar effect on O_2 consumption and proton production. Figure 5 combines all data for inhibition of MnO_2 production, O_2 consumption, and proton formation by azide. Azide inhibited these three activities to the same extent.

DISCUSSION

Whole cells of *L. discophora* SS1 oxidized Mn^{2+} to MnO_2. Although it has been suggested that in batch culture MnO_2 is only formed when cells have reached the stationary phase of growth (14), we found that cells in the early exponential phase were also able to oxidize Mn^{2+}. Similar findings with *L. discophora* have been reported by van Veen (21) and Adams and Ghiorse (1).

Not only cells but also spent culture medium were able to oxidize Mn^{2+}, as also noted by Ghiorse (13). Both growing and resting cells released their Mn^{2+}-oxidizing activity into the medium. The release of the Mn^{2+}-oxidizing activity into the medium made it possible to characterize the process without the interference of cellular metabolism. Our results strongly indicate that Mn^{2+} oxidation in spent culture medium is catalyzed by a protein or a substance with a considerable protein content. The activity was affected by NaN_3, pronase, SDS, high salt concentrations, $HgCl_2$, and high temperatures. It had a sharp pH optimum. At least two Mn^{2+}-oxidizing products with apparent molecular weights of 110K and 85K were detected by SDS-polyacrylamide gel electrophoresis. Both could be stained with Coomassie blue and silver. The 110K and 85K products may represent distinct molecular species. Alternatively, the 85K product may be a breakdown moiety of the 110K molecule generated by proteolytic enzymes excreted by the bacteria or liberated by lysed cells. A proteolytic degradation product still able to oxidize Mn^{2+} was produced by the action of trypsin. The inhibition of Mn^{2+} oxidation by phosphate and Tris is not easily explained. In general, substances that interfere with manganese chemistry inhibit the oxidation process (20);

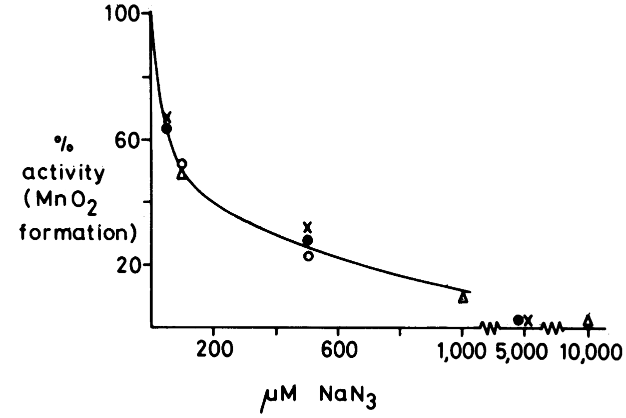

FIG. 5. Effect of azide on manganese oxide production (×, △), oxygen consumption (○), and proton liberation (●) by spent culture medium. MnO_2 formation: 100% activity was 5.7 (×) or 1.0 (△) nmol of MnO_2 formed per ml per min; O_2 consumption: 100% activity was 6.5 nmol of O_2 per ml per min; proton production: 100% activity was 17.4 nmol of H^+ per ml per min. Activities were determined in the same (× and ●) and in different (○ and △) preparations of spent culture medium.

phosphate may compete with the Mn^{2+}-oxidizing protein for the Mn^{2+} ion. Tris has no Mn^{2+}-complexing abilities (20); it may affect the oxidizing protein in an as yet unknown manner.

An important question is whether the Mn^{2+}-oxidizing protein is a true catalyst. Since molecular oxygen was consumed during the oxidation process in a stoichiometric reaction with Mn^{2+}, it is unlikely that the protein was simultaneously reduced. The protein coprecipitated with the oxide formed, but the oxidizing activity could be partially recovered by dissolving the precipitate with a reducing agent. The loss of activity during this procedure may very well have been due to the numerous filtration steps necessary to remove the reducing agent. In some cases we noted that not all of the added Mn^{2+} was oxidized (e.g., Fig. 1A). This would not be expected if an enzyme were involved in Mn^{2+} oxidation. A possible explanation may be that the Mn^{2+}-oxidizing factor loses its activity when it is heavily encrusted with MnO_2. Such an observation was made with the Mn^{2+}-oxidizing spores of a marine *Bacillus* species (5). In preparations in which 200 μM Mn^{2+} was completely oxidized, the oxidation apparently obeyed Michaelis-Menten kinetics in the range of 0 to 200 μM Mn^{2+}, with an apparent K_m of 13 μM Mn^{2+} (unpublished observations).

Although our data on the consumption of O_2 and the production of protons during Mn^{2+} oxidation roughly agree with the equation $Mn^{2+} + 0.5O_2 + H_2O \rightarrow MnO_2 + 2H^+$, the process is probably more complicated. Since the initial rate of proton production exceeds that of MnO_2 formation by a factor far more than 2, one of the first steps in the oxidation process may be the adsorption of Mn^{2+} ions to incipiently formed hydrated oxide, with concomitant proton release (9, 10). The lag phase generally observed in Mn^{2+} oxidation (Fig. 1 and 4) may represent the formation of this oxide without the stimulating effect of adsorption. Clearly the processes of MnO_2 formation, proton production, and O_2 consumption are closely linked or ordered, since NaN_3 inhibited all of them to the same extent.

The fact that Mn^{2+} oxidation by *L. discophora* SS1 is catalyzed by at least one homogeneous protein may permit the investigation of Mn^{2+} oxidation in this species on a molecular level. This investigation has to await the isolation of the Mn^{2+}-oxidizing factor(s) on a preparative scale.

ADDENDUM

After submission of this paper, the editor brought other recent results to our attention. Ghiorse and Adams detected a manganese-oxidizing protein with an apparent molecular weight of 110K in the spent culture medium of *L. discophora* SS1 (W. C. Ghiorse, Biotechnol. Bioeng. Symp. **16**:141–148, 1986). This result is in complete agreement with our data.

ACKNOWLEDGMENTS

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