JOURNAL OF BACTERIOLOGY, July 1986, p. 30–34
0021-9193/86/070030-05$02.00/0

Vol. 167, No. 1

Manganese Reduction by a Marine *Bacillus* Species

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Received 12 December 1985/Accepted 3 April 1986

Mature dormant spores of marine *Bacillus* sp. strain SG1 catalyze the oxidation of Mn(II) to MnO_2. We report that vegetative cells of the same strain reduced MnO_2 under low-oxygen conditions. The rate of reduction was a function of cell concentration. The process had a pH optimum of 7.5 to 8.0 and was inhibited by $HgCl_2$, by preheating of the cells at 80°C for 5 min, by antimycin A, and by *N*-heptyl-hydroxy-quinoline-*N*-oxide. At a nonlimiting O_2 concentration, little MnO_2 reduction was observed. Under these conditions, the process could be induced by the addition of NaN_3. Spectrophotometric analysis of the *Bacillus* cells indicated the presence of type *b* and *c* cytochromes. Both types can be oxidized in situ by addition of MnO_2 to the cells.

It is well known that bacteria from many genera catalyze the conversion of manganese between valence states, mainly between +II and +IV (4, 5, 8, 9). In some cases the oxidation of Mn(II) or the reduction of Mn(IV) results from changes in the pH or E_h of the environment through bacterial metabolism (indirect transformation [5, 9]). On the other hand, many organisms produce specialized macromolecules which catalyze the redox reactions (direct catalysis), and in some cases it has been shown that enzymes are involved (3, 6, 12).

Rosson and Nealson (11) demonstrated oxidation of Mn(II) by mature, dormant spores of a marine *Bacillus* species, strain SG1. Vegetative cells of this strain did not oxidize Mn(II). The data suggested that manganese was oxidized by a spore component, possibly an exosporium or spore coat protein. Tebo (Ph.D. thesis, University of California, San Diego, 1983) discussed the possible function of the Mn(II)-oxidizing capacity of the (metabolically inert) spores. He hypothesized that the presence of MnO_2 on the spore surface might confer some advantage to the species on germination in a low-oxygen or anaerobic environment. The Mn(IV) might be used as an electron acceptor in respiration. In the absence of oxygen, some manganese-reducing organisms may use manganese oxides as electron acceptors (2). In a *Bacillus* species isolated from manganese nodules, the presence of an inducible manganese oxide reductase was demonstrated. It was suggested that this enzyme also operated under aerobic conditions (5, 12). Bromfield and David (1) described a soil *Arthrobacter* species which oxidized Mn(II) in the presence of oxygen but reduced the oxide formed under certain anaerobic conditions, the reduction being enhanced by exogenous electron donors.

In the present study, we investigate the reduction of manganese oxide by vegetative cells of *Bacillus* sp. strain SG1. Special attention is given to the possibility that this process is coupled to the electron transport system.

MATERIALS AND METHODS

Organism and its cultivation. *Bacillus* sp. strain SG1, isolated by Nealson and Ford (10), was grown at room temperature on a modified K medium (11) containing, per liter of 50 mM Tris in 80% natural seawater (pH 7), 2 g of peptone (Difco Laboratories), 0.5 g of yeast extract (Difco), 10 μg of ferric EDTA, and 100 μg of $MnCl_2 \cdot 4H_2O$ (filter sterilized). Cells were harvested in the logarithmic phase by centrifugation (10 min, 10,000 × *g*), washed twice with K medium from which $MnCl_2$ was omitted (K^- medium), and suspended in K^- medium.

Determination of cell concentration. Cell concentrations were measured by reading the optical density of cell suspensions at 660 nm (OD_{660}). To correlate the OD_{660} with the number of cells, cells were counted in a Bürker counting chamber. The relation between the OD_{660} and cell number was linear up to an OD_{660} of at least 1, corresponding to $3 \times 10^8 \pm 1 \times 10^8$ cells per ml.

Determination of manganese reduction. To measure the reduction of manganese, a suspension of manganese oxide was added to vegetative cells. The manganese oxide suspension was prepared by reduction of 20 mM $KMnO_4$ with an equal volume of 30 mM Na_2SO_3 in an alkaline environment. Alternatively, the $KMnO_4$ was mixed with an equal volume of 30 mM $MnCl_2$. The oxide formed was centrifuged and washed three times with distilled water. After each centrifugation, the oxide was suspended by ultrasonication. The exact elemental composition of the manganese oxide was not determined. The ratio of Mn to O may have been slightly lower than 2. The oxide will be hereafter referred to as MnO_2. No difference in behavior was noted between the oxides prepared by the two procedures.

MnO_2 was added to suspensions of cells in K^- medium (cell concentrations varied between 1×10^8 and 20×10^8 cells per ml, as indicated below and in the figure legends), until a final concentration of about 100 μM was reached. At times specified below, duplicate samples of 0.1 ml were taken and added to 0.5 ml of 0.04% Leuco Berbelin blue (LBB) in 0.25% acetic acid to determine the amount of MnO_2 (7). After mixing and removal of cells by centrifugation, the A_{620} was read. $KMnO_4$ was used as a standard. The LBB assay followed Beer's law over the A_{620} range of 0 to 1.5. A concentration of 40 μM $KMnO_4$ (equivalent to 100 μM MnO_2) resulted in an A_{620} of 1.2 in the LBB assay.

Oxygen consumption by vegetative cells. Oxygen consumption was measured by using a Clark oxygen electrode in a closed cell with a volume of 1 ml at 25°C.

Analysis of cytochromes in vegetative cells. Spectrophotometric analyses were performed by using an Aminco DW-2 UV/Vis spectrophotometer equipped with a beam scrambler for measurements of light-scattering samples. Spectra were recorded at room temperature by using a Thunberg cuvette against a fixed reference wavelength of 570 nm. Spectra of frozen samples were recorded at 77°K. Bacteria were incu-

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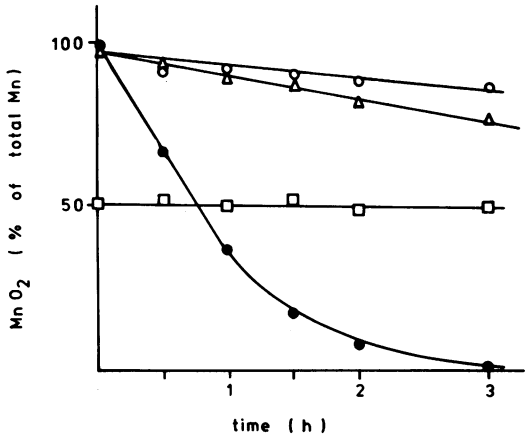

FIG. 1. MnO_2 reduction by vegetative cells of *Bacillus* sp. strain SG1. In three experiments, MnO_2 was added to the samples until a final concentration of 100 μM was reached. At specified times, the MnO_2 in the suspensions was measured with the LBB assay. The amount of MnO_2 is expressed as the percentage of the total manganese added at time zero. Symbols: ●, MnO_2 concentration in a suspension of vegetative cells (10^9 cells per ml); ○, MnO_2 concentration in the uninoculated assay medium or spent culture medium supernatant fluid; △, MnO_2 concentration in a cell suspension sparged with oxygen; □, MnO_2 concentration in a suspension of 50 μM MnO_2 in uninoculated K^- medium, which contained 50 μM $MnCl_2$ and was sparged with oxygen.

bated in K^- medium at 2×10^{10} cells per ml for 10 min to allow the endogenous reduction of cytochromes. They were kept anaerobic by a continuous flow of argon directed at the sample surface. MnO_2 was added to the suspension, and spectra were recorded at 10-min intervals.

RESULTS

Reduction of manganese by cells of *Bacillus* sp. strain SG1. Spores of *Bacillus* sp. strain SG1 are able to oxidize Mn^{2+} to MnO_2. The product remains bound to the spore surface (11). We found that spores which had been stored for longer periods of time tended to germinate, even in the absence of nutrients. On such occasions, it was noted that the MnO_2 formed by spores was dissolved, suggesting MnO_2 reduction by vegetative cells (Tebo, Ph.D. thesis). Therefore, experiments were performed to check whether vegetative cells are indeed capable of manganese reduction.

Cells were grown in K medium and harvested in the logarithmic phase of growth (about 10^8 cells per ml) by centrifugation. Subsequently, they were suspended in K^- medium at a final concentration of 10^9 cells per ml. Under these conditions the cells consumed oxygen at a rate of 43 μmol/min. Consequently, all oxygen was removed from the culture within 6 min. The cell suspension also remained oxygen depleted when it was continuously stirred in open air (results not shown). Apparently the rate of oxygen consumption exceeded that of oxygen dissolution in the medium. When MnO_2 was added to this cell suspension, it was completely reduced within 3 h (Fig. 1). Cells did not divide during this period. Cells suspended in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) in 80% natural seawater (pH 7.0) also reduced MnO_2 albeit at a slightly lower rate (results not shown). Addition of glucose or succinate (to a concentration of 100 mM) did not affect the reduction rate. Both the K^- medium and the medium in which the cells had been previously grown showed only slight reducing activity (Fig. 1). This activity was increased neither by expulsion of oxygen by sparging the media with nitrogen nor by previous incubation of cells in the media at a high concentration (10^9 cells per ml) for 2 h. These results indicate that cells of *Bacillus* sp. strain SG1 were capable of MnO_2 reduction. The activity was apparently associated with the cells and not caused by an excretion product. Due to the rapid O_2 consumption, the cells created sufficiently anaerobic conditions to permit MnO_2 reduction. This observation is sustained by the fact that sparging the cell suspensions with nitrogen did not increase the rate of MnO_2 reduction. When, however, oxygen was continuously dispersed in the cell cultures, MnO_2 reduction was strongly inhibited (Fig. 1). Replacement of oxygen by nitrogen after 90 min caused MnO_2 reduction to start at a rate similar to that of the anaerobic cells (results not shown). This indicates that the inhibition of reduction was not due to oxygen poisoning of the cells. Under conditions in which oxygen is dispersed in the cultures, Mn^{2+} ions produced by MnO_2 reduction by the cells may be chemically reoxidized. The latter reaction may be catalyzed by the manganese oxide (autocatalysis). The possibility that autocatalytic Mn(II) oxidation masked the reduction process was excluded in the following control experiment. Oxygen was dispersed in a suspension of MnO_2 in K^- medium containing an equimolar amount of $MnCl_2$ (50 μM). Oxidation of Mn^{2+} was not observed (Fig. 1).

Factors affecting manganese reduction. The rate of MnO_2 reduction increased with increasing cell concentration (Fig. 2A, inset). The relation between the reduction rate (in micromoles of MnO_2 per hour) and the cell concentration was linear at least up to 7.8×10^8 cells per ml, the reduction rate at 0 cells per ml representing the activity of the medium itself (Fig. 2A and Fig. 1). The reduction rate (at 10^9 cells per ml) was plotted as a function of the pH of the assay medium (Fig. 2B). Although nonbiological reduction was facilitated by lowering the pH below 7, the process reached an optimum at a pH between 7.5 and 8.0 in the presence of cells of *Bacillus* sp. strain SG1. It should be kept in mind that, at a pH of 8.5 or higher, the reducing activity would be difficult to quantify. Under those conditions the Mn(II) formed would be rapidly removed by auto-oxidation (11).

Cells which were preheated at 80°C for 5 min completely lost their ability to reduce manganese oxide. The reduction was also inhibited by $HgCl_2$ at a concentration of 0.01% (Fig. 3). Addition of $HgCl_2$ (final concentration, 0.01%) to an anoxic cell suspension that was actively reducing MnO_2 caused an almost instantaneous cessation of the MnO_2 reduction (Fig. 3). These effects and the apparent pH optimum for MnO_2 reduction indicate that the process was catalyzed by the cells.

Involvement of the electron transport system in MnO_2 reduction. When cells of *Bacillus* sp. strain SG1 were treated with lysozyme (2 mg/ml for 2 h at room temperature), spheroplasts were produced. After centrifugation, these spheroplasts (suspended in K^- medium) reduced MnO_2 at the same rate as did the original cell suspension, whereas the supernatant was inactive. Because it is less likely that the solid MnO_2 particles are able to pass through the plasma membrane, the latter is probably the site where the reducing activity is located, suggesting the involvement of the electron transport system of the bacteria. Therefore, the effects of three electron transport inhibitors, antimycin A, *N*-heptyl-hydroxy-quinoline-*N*-oxide (HOQNO), and NaN_3, on the process were studied. First, their interference with

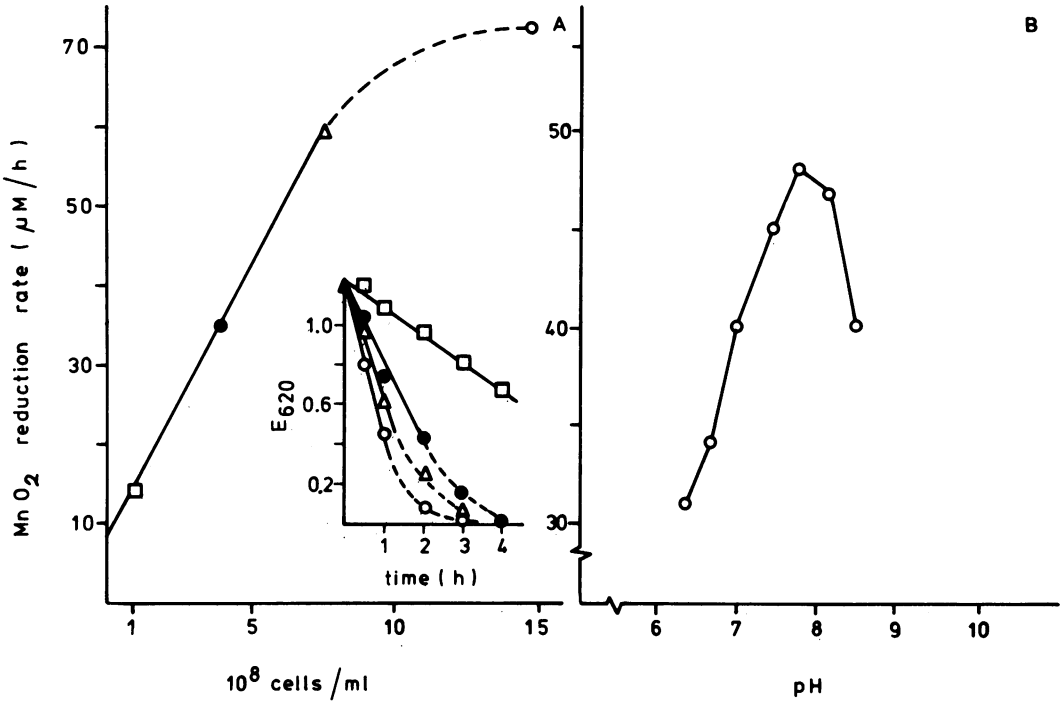

FIG. 2. Effect of cell concentration and pH on MnO_2 reduction. Cultures were incubated with 100 μM MnO_2 at different cell concentrations (A) or at different pH values of the assay medium (B) (10^9 cells per ml). MnO_2 concentrations were measured as described in the legend to Fig. 1. The MnO_2-reducing activity is expressed as the initial reduction velocity (v) in micromoles of MnO_2 per hour. The rate was calculated from the decrease of the A_{620} per hour in the linear part of the plots (inset). For further experimental details, see Materials and Methods.

electron transfer to oxygen was measured. The inhibitors were added to cell suspensions of 10^9 cells per ml in K^- medium (oxygen consumption rate, 43 μmol/min; see above). Addition of antimycin (final concentration, 20 μg/ml) almost instantaneously reduced the rate of oxygen uptake by cells to 10% of the original level. In the presence of 20 μM HOQNO, 20% of the original activity was left. At increasing concentrations of NaN_3, the rate of oxygen uptake gradually decreased to 40% of the initial rate at about 20 mM NaN_3. At concentrations higher than 20 mM, no further effect was observed (Fig. 4). This observation suggests that *Bacillus* sp. strain SG1 contains at least two terminal oxidases, one of which is insensitive to azide. The effect of the electron transport inhibitors on MnO_2 reduction is illustrated in Fig. 5. Because HOQNO had an effect similar to that of antimycin, only the results obtained with the latter substance are depicted. Antimycin reduced the rate of MnO_2 reduction in O_2-depleted cell suspensions by 40% at a concentration of 20 μg/ml (Fig. 5A). A similar result was obtained in the presence of 20 μM HOQNO. Both substances clearly inhibited MnO_2 reduction less strongly than the consumption of oxygen. Both antimycin and HOQNO were added in small volumes of concentrated solutions in ethanol. Addition of equal volumes of the solvent affected neither the rate of O_2 uptake nor that of MnO_2 reduction. NaN_3 (20 mM) had no effect on the reduction in anaerobic cell suspensions, but it induced the process in cell cultures sparged with oxygen (Fig. 5B). It should be noted that the rate of MnO_2 reduction measured under the latter conditions varied in different cell batches from about 80 to 50% the rate in anaerobic cell suspensions.

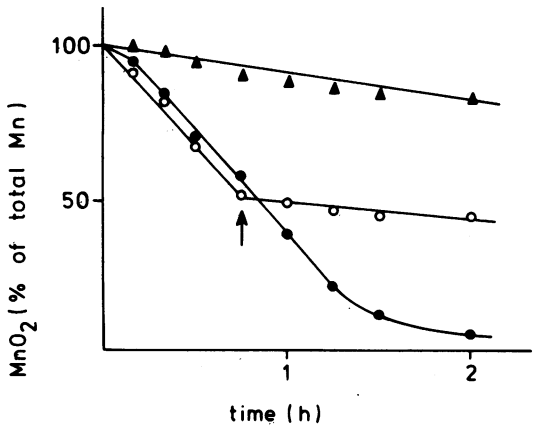

FIG. 3. Inhibition of MnO_2 reduction by $HgCl_2$. Cell suspensions of 10^9 cells per ml were incubated with 100 μM MnO_2. $HgCl_2$ was added to a final concentration of 0.01% (wt/vol) at time zero (▲) or at 45 min (○), and results were compared with those obtained in a control cell suspension (●). MnO_2 was measured as described in the legend to Fig. 1.

The inhibition of oxygen consumption and MnO_2 reduction by antimycin A and HOQNO points to the involvement of cytochromes in both processes. The presence of cytochromes in *Bacillus* sp. strain SG1 cells was confirmed by recording a spectrum of anoxic cells from 500 to 600 nm. Two absorption maxima at 550 and 559 nm were observed, which are characteristic for cytochrome *c* and *b*, respectively (Fig. 6, line a and inset). When MnO_2 was added to this cell suspension, both peaks decreased to a steady-state level (Fig. 6, line b). When a spectrum was recorded after a short interval, the absorptions had increased again (Fig. 6,

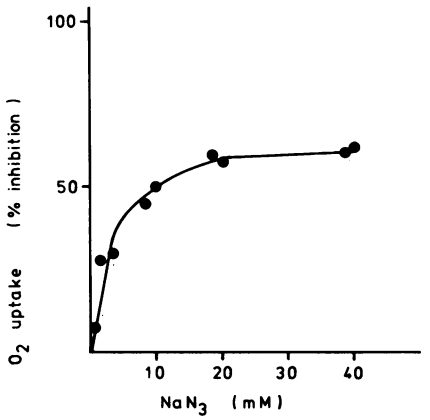

FIG. 4. Inhibition of oxygen consumption by NaN_3. Oxygen consumption was measured with an oxygen electrode in cell suspensions containing different concentrations of NaN_3. The percent inhibition at a given NaN_3 concentration is defined as 100 minus the percent uptake rate with no NaN_3. The cell concentrations were 10^9 cells per ml. The 100% rate of oxygen consumption was 43 μmol/min.

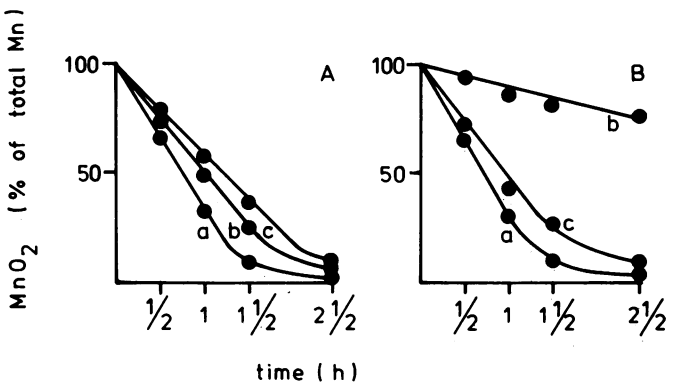

FIG. 5. Effect of antimycin and NaN_3 on the MnO_2 reduction. MnO_2 reduction was measured as described in the legend to Fig. 1. (A) Antimycin was added to a cell suspension until one of the following concentrations was reached: 0 μg/ml (a), 5 μg/ml (b), or 20 μg/ml (c). (B) MnO_2 reduction by cells in the presence of 20 mM NaN_3 (a), by cells sparged with oxygen (b), or by cells sparged with oxygen in the presence of 20 mM NaN_3 (c). Cell concentrations, 10^9 cells per ml.

line c). At this moment, all MnO_2 had been reduced, as was determined with LBB. Apparently the presence of MnO_2 in an O_2-depleted cell culture caused the oxidation of cytochromes *c* and *b*.

DISCUSSION

Bacillus sp. strain SG1, a marine bacterial species isolated from a near-shore manganese nodule (10), is capable of different manganese conversions, depending on its life cycle. The inert dormant spores catalyze the oxidation of Mn^{2+} to MnO_2. The vegetative cells, on the other hand, do not have the oxidizing capacity (11) but are able to reduce MnO_2 under appropriate conditions. An important question is whether these two processes are functionally related. Can the oxide accumulated in the dormant life stage be used as a terminal electron acceptor in primary metabolism? An organism carrying an alternative electron acceptor might successfully compete with species depending on oxygen only.

The results presented in this paper suggest that components of the electron transport system of vegetative cells are involved in manganese reduction. When the oxygen concentration was nonlimiting, almost no MnO_2 reduction was observed. Under these conditions, the cells preferentially transferred electrons to oxygen. Exhaustion of the oxygen supply by the cells induced reduction of the oxide. MnO_2 was also reduced when electron transfer to oxygen was partially inhibited by NaN_3. The fact that the rate of reduction was variable under these conditions may be explained by the presence of at least one terminal oxidase which displays low sensitivity to NaN_3. The rate of MnO_2 reduction would partially depend on the activity of this enzyme, which might vary in different cell batches.

Spectrophotometric analysis of *Bacillus* sp. strain SG1 cells revealed the presence of *b*- and *c*-type cytochromes. Both were oxidized after the addition of MnO_2 under anoxic conditions. The fact that both cytochromes were reduced again after the consumption of the oxide indicates that the MnO_2 was responsible for the observed oxidation. This result explains the inhibition of MnO_2 reduction by antimycin A and HOQNO, both known to block electron transfer from cytochrome *b* to cytochrome *c*. The fact that the inhibitors affected the MnO_2 reduction less strongly than the O_2 consumption could imply that the oxide is able to react with both types of cytochromes.

If primary metabolism were involved in MnO_2 reduction, addition of exogenous substrates would stimulate the process. But neither glucose nor succinate was found to enhance the reduction rate. Sufficient supplies of endogenous substrates were probably available. In principle, to study this possibility, the *Bacillus* cells should be submitted to starvation before the addition of glucose or succinate. This was impossible, however, because the cells would sporulate under those conditions.

In some respects, the results presented here are similar to those obtained by Trimble and Ehrlich (12) and Ghiorse and Ehrlich (6). These authors also reported on a marine *Bacillus* species capable of manganese reduction. As in our case, strict anaerobiosis was not a prerequisite for Mn(IV) reduction by this species. The authors even stated that the kinetics

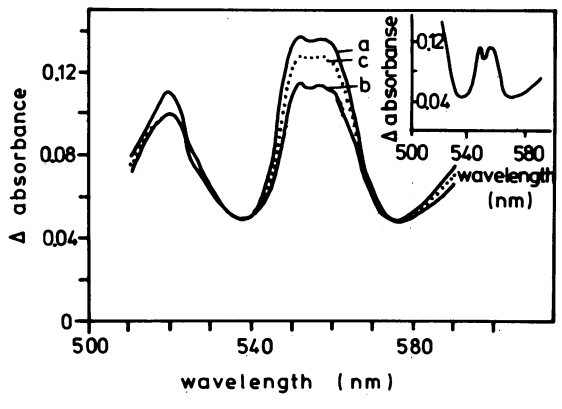

FIG. 6. Absorption spectra (500 to 600 nm) minus A_{570} of vegetative cells. A suspension of 2×10^{10} cells in K^- medium was incubated for 10 min at room temperature before the first spectrum was recorded (line a). MnO_2 was added to a concentration of 100 μM, and spectra were taken after 10 and 20 min (line b) and after 30 min (line c). Inset, Spectrum of a cell suspension without MnO_2 recorded at 77°K, resulting in a better resolution and a slight shift of the absorption peaks.

of the process were the same under aerobic and anaerobic conditions. However, in their experiments, the rate of oxygen consumption was not determined, and the MnO_2 reduction activity was assessed by measuring the Mn^{2+} released after 3 h. Therefore, no conclusion can be drawn from these results with regard to the influence of O_2 tensions. The authors suggested that the organism can use MnO_2 as a terminal electron acceptor and that the electron transfer to MnO_2 is mediated by a MnO_2 reductase, which is inducible by Mn^{2+} ions. It is unknown whether a reductase plays a role in the reduction of MnO_2 by vegetative cells of *Bacillus* sp. strain SG1. In a preliminary experiment, no difference in reducing activity was noted between cells grown in K medium and cells grown in K^- medium. This indicates that an induction mechanism as proposed by Trimble and Ehrlich (12) was not operating in *Bacillus* sp. strain SG1. If electron transfer to MnO_2 is mediated by an enzyme, the latter would probably be located at the plasma membrane. The localization of the MnO_2-reducing activity may be studied by measuring the reduction of MnO_2 by membrane vesicles supplied with suitable exogenous electron donors under anaerobic conditions. If the reduction is coupled to the electron transport system, it could be expected that *Bacillus* sp. strain SG1 would be able to germinate and grow in the presence of MnO_2 under anaerobic conditions. The reduction of MnO_2 by vegetative cells, however, was a slow process as compared with the consumption of oxygen. Therefore, determination of growth resulting from the use of MnO_2 as an electron acceptor requires careful experimentation.

ACKNOWLEDGMENTS

We thank W. N. M. Reijnders, Free University of Amsterdam, for his technical assistance with the spectrophotometrical analyses. We are grateful to Leendert Bosch, Peter Westbroek, and Reinhardt Rosson for their helpful discussions and suggestions and for critically reading the manuscript.

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