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Partial Purification and Characterization of Manganese-Oxidizing Factors of *Pseudomonas fluorescens* GB-1

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The Mn^{2+}-oxidizing bacterium *Pseudomonas fluorescens* GB-1 deposits Mn oxide around the cell. During growth of a culture, the Mn^{2+}-oxidizing activity of the cells first appeared in the early stationary growth phase. It depended on the O_2 concentration in the culture during the late logarithmic growth phase. Maximal activity was observed at an oxygen concentration of 26% saturation. The activity could be recovered in cell extracts and was proportional to the protein concentration in the cell extracts. The specific activity was increased 125-fold by ammonium sulfate precipitation followed by reversed-phase and gel filtration column chromatographies. The activity of the partly purified Mn^{2+}-oxidizing preparation had a pH optimum of circa 7 and a temperature optimum of 35°C and was lost by heating. The Mn^{2+}-oxidizing activity was sensitive to NaN_3 and $HgCl_2$. It was inhibited by KCN, EDTA, Tris, and *o*-phenanthroline. Although most data indicated the involvement of protein in Mn^{2+} oxidation, the activity was slightly stimulated by sodium dodecyl sulfate at a low concentration and by treatment with pronase and V8 protease. By polyacrylamide gel electrophoresis, two Mn^{2+}-oxidizing factors with estimated molecular weights of 180,000 and 250,000 were detected.

The oxidation of manganese (Mn^{2+}) by microbes has been documented since the beginning of this century (3). Many bacterial species with the ability to oxidize Mn^{2+} occur in soils and in fresh- and marine waters (16). Studies of these bacteria have been concerned with their roles in the geochemical cycling of manganese in nature (16), their possible participation in the formation of manganese nodules (12), and the biochemical characterization of the Mn^{2+}-oxidizing processes (1, 4, 10–12). Several reports described the involvement of proteinaceous macromolecules in Mn^{2+} oxidation by bacterial species of *Pseudomonas* (11, 13), *Citrobacter* (11), *Arthrobacter* (12), *Leptothrix* (1, 4), and *Bacillus* (10). In all cases the oxidation of Mn^{2+} appeared to be catalyzed, suggesting the involvement of enzymes. In none of these cases, however, have the Mn^{2+}-oxidizing macromolecules been purified to such an extent as to allow their biochemical and structural characterization. Recently, genes implicated in Mn^{2+} oxidation have been identified in a marine *Bacillus* sp. (18) and in *Leptothrix discophora* (8). Some of the genes encoded proteins resembling multicopper oxidases. These proteins were postulated to be directly involved in Mn^{2+} oxidation, but unambiguous evidence that they represent the Mn^{2+}-oxidizing factors has not been presented. Consequently, no Mn^{2+}-oxidizing factor has been characterized in detail, and the physiological significance of bacterial Mn^{2+} oxidation has been obscure.

Pseudomonas fluorescens GB-1 is an Mn^{2+}-oxidizing freshwater bacterium. The Mn^{2+}-oxidizing activity is associated with the cells. In a previous study it was demonstrated that an Mn^{2+}-oxidizing factor(s) could be solubilized by disruption of the cells (7). The Mn^{2+}-oxidizing activity was detected (semiquantitatively) with a dot blot assay. In this study we characterized the nature of the Mn^{2+}-oxidizing activity, and we describe the partial purification of Mn^{2+}-oxidizing proteins isolated from cell extracts of this Mn^{2+}-oxidizing *Pseudomonas* strain.

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MATERIALS AND METHODS

Organism and its cultivation. The Mn^{2+}-oxidizing bacterial isolate was kindly provided by K. H. Nealson, University of Wisconsin, Milwaukee. The culture medium was identical to that described for *L. discophora* (4). The bacteria were routinely grown at 23°C in 250 ml of medium in a 1-liter Erlenmeyer flask with continuous shaking on a reciprocating shaker (85 strokes/min).

Electron microscopy. Manganese-oxidizing bacteria were examined by transmission electron microscopy (TEM) to detect the site of oxide deposition. Cells from early-stationary-phase cultures were harvested by centrifugation at $10,000 \times g$, washed once with 10 mM HEPES-NaOH buffer (pH 7.4), suspended in HEPES buffer (control) or in HEPES buffer containing 500 μM $MnCl_2$, and incubated overnight. The cells were fixed with osmium tetraoxide for 12 h and dehydrated through a series of ethanol solutions. They were then embedded in Epon 812. Thin sections were poststained with uranyl acetate and lead citrate and observed at 80 kV with a JEM-100CX II microscope (JEOL). The presence of manganese in the deposits was qualitatively established with an analytical electron microscope (TEM-2000FX, EDS; JEOL). Prefixation with glutaraldehyde was omitted because the MnO_2 deposits were dissolved by this treatment.

Biochemical identification of bacteria. The bacteria were identified with a clinical kit (Pile tube no. 2; Eiken Chemical Co.) for identification of nonfermentative, gram-negative rods.

Assays of Mn^{2+}-oxidizing activity. The Mn^{2+}-oxidizing activity was determined colorimetrically with Leukoberbelin blue according to the method of Krumbein and Altmann (14) as modified by Boogerd and de Vrind (4). Leukoberbelin blue is oxidized by manganese of oxidation states of +3 and higher, producing a blue color. The assays were started by adding $MnCl_2$ to a final concentration of 100 μM. At regular intervals samples of 0.2 ml were added to 1 ml of 0.04% Leukoberbelin blue in 45 mM acetic acid, and the absorbance was read at 620 nm. $KMnO_4$ was used as a standard. The Leukoberbelin blue assay followed Beer's law over the absorption range of 0 to 1.5. A concentration of 40 μM $KMnO_4$ (equivalent to 100 μM MnO_2) resulted in an absorption of 1.2 in the assay. Leukoberbelin blue was synthesized as described by Altmann (2). For measurements of the activity of bacterial cells, cells were harvested by centrifugation and suspended in 10 mM HEPES buffer (pH 7.4) to an optical density at 660 nm ($0D_{660}$) of 1.0, unless stated otherwise. Prior to the absorbance measurements, the cells were removed from the samples by centrifugation. The spent culture medium was screened for possible release of Mn^{2+}-oxidizing activity from the cells. Prior to the oxidation assay, the medium was filtered through a bacterial filter (pore size, 0.2 μm). The activities in cell extracts and partially purified Mn^{2+}-oxidizing preparations were measured after dilution of the samples in appropriate buffers or solutions as indicated below. All measurements were performed at room temperature unless stated otherwise.

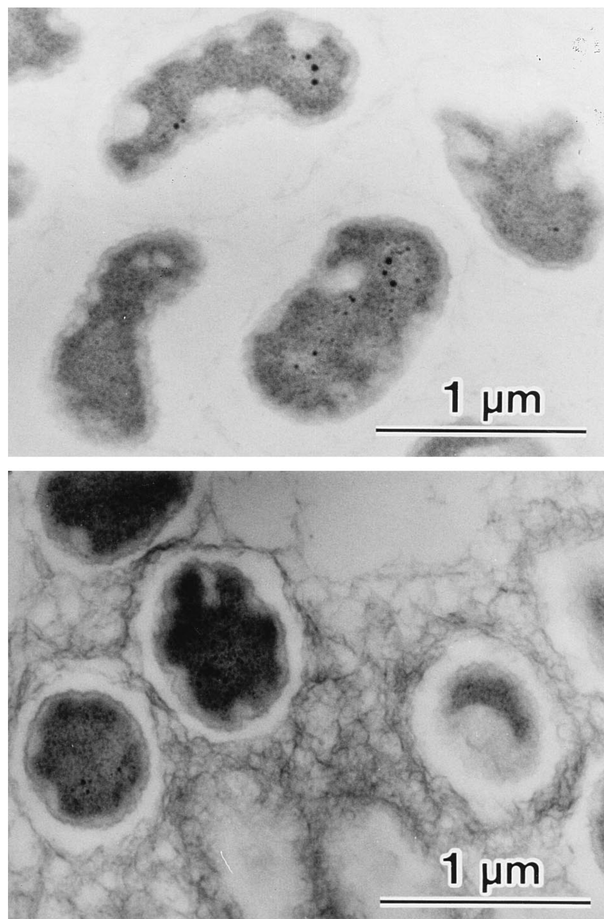

FIG. 1. Transmission electron micrographs showing the site of manganese deposition. Cells in the early stationary phase of growth were incubated overnight in a HEPES buffer (pH 7.4) without $MnCl_2$ (upper micrograph) (control) or with 500 μM $MnCl_2$ (lower micrograph). Staining was with lead citrate and uranyl acetate.

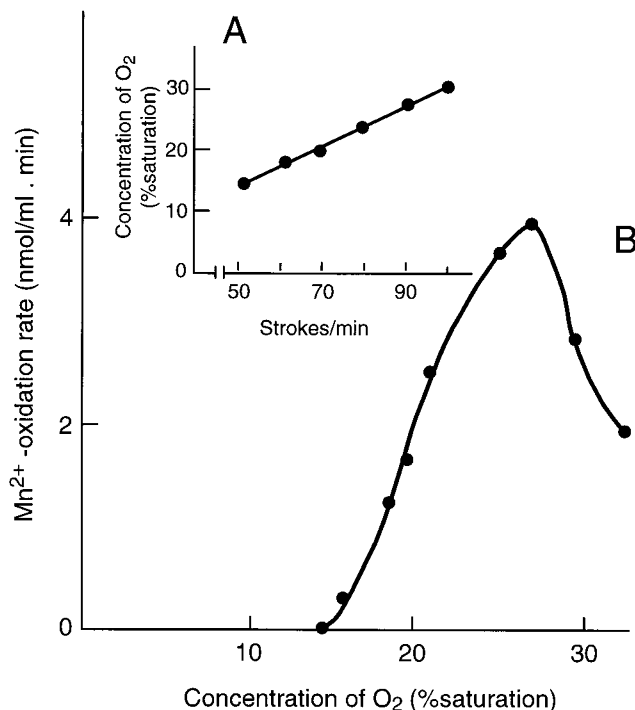

FIG. 2. Relation between the Mn^{2+}-oxidizing activity of cells in the early stationary growth phase and the O_2 concentration in the culture in the late logarithmic growth phase. (A) O_2 concentration in a late-logarithmic-phase culture as a function of the rate of shaking of the culture. (B) Mn^{2+}-oxidizing activity of cells in the early stationary phase as a function of the O_2 concentration in the culture in the late logarithmic phase.

Determination of oxygen content in the culture medium. The change in oxygen concentration in the culture during bacterial growth was measured in 100-ml cultures in 500-ml flasks at 20°C with a Clark oxygen electrode (type 4004; Yellow Springs Instrument Co., Yellow Springs, Ohio). The absolute amount of dissolved oxygen in the culture medium was measured with Leukoberbelin blue by the method of Altmann (2) and expressed as the degree of saturation (percent) at 20°C. The method is based on the redox reaction of $Mn(OH)_2$ with O_2 in an alkaline environment to form hydrated manganese oxides (referred to as MnO_2). The amount of MnO_2 formed is subsequently determined in an acidic environment with Leukoberbelin blue. The oxygen determinations were performed in sealed vessels as described by Altmann (2) to exclude oxygen from the air.

Preparation of cell extracts. Bacterial cells in the early stationary growth phase were harvested by centrifugation and suspended in 10 mM HEPES buffer (pH 7.4) to an OD_{660} of 1.0. They were disrupted by ultrasonication at 20 kHz for 30 min at 30-s intervals. All procedures were performed at 4°C. The homogenate was centrifuged at 25,000 × *g* for 30 min, and the supernatant (Sup25k) and the precipitate (Ppt25k) were collected. The Ppt25k was resuspended in the original volume of HEPES buffer. To determine whether Mn^{2+}-oxidizing activity was associated with particulate material, the Sup25k was recentrifuged at 100,000 × *g* for 60 min. The supernatant (Sup100k) was collected, and the precipitate (Ppt100k) was resuspended in the original volume of HEPES buffer.

Purification of Mn^{2+}-oxidizing components. Three liters of early-stationary-phase cultures was harvested by centrifugation and suspended in 200 ml of HEPES buffer. A cell extract (Sup25k) was prepared as described above. To the 200 ml of the cell extract, solid $(NH_4)_2SO_4$ was added to 30% saturation (35.2 g), and the suspension was stirred for 1 h. Precipitated proteins were collected by centrifugation at 12,000 × *g* for 20 min. The precipitated proteins were dissolved in 75 ml of HEPES buffer containing 0.5 M $(NH_4)_2SO_4$ and were subjected to reversed-phase chromatography on a butyl-Toyopearl 650 column (XK 26/40; Pharmacia) equilibrated with HEPES–0.5 M $(NH_4)_2SO_4$. The column was rinsed with HEPES–0.5 M $(NH_4)_2SO_4$ and subsequently eluted at 2.0 ml/min with a linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in HEPES buffer. Fractions of 5 ml were collected. Samples of each fraction were diluted 10 times in HEPES buffer and screened for Mn^{2+}-oxidizing activity [the Mn^{2+}-oxidizing activity was not inhibited up to at least 0.1 M $(NH_4)_2SO_4$]. The fractions containing Mn^{2+}-oxidizing activity were pooled, and the proteins were precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. The proteins were collected by centrifugation at 100,000 × *g* for 30 min, dissolved in 5 ml HEPES buffer, and dialyzed against HEPES buffer for 24 h. The dialyzed butyl-Toyopearl fraction was subjected to gel filtration on a Sephacryl S 300 column (C26/100; Pharmacia). The column was eluted with HEPES buffer containing 0.1 M $(NH_4)_2SO_4$ at 2 ml/min, and fractions of 5 ml were collected and screened for Mn^{2+}-oxidizing activity as described above. The fractions with peak Mn^{2+}-oxidizing activity were pooled and concentrated to 5 ml by ultrafiltration with a Filtron cell system (molecular weight limit, 100,000). The filtrates did not contain Mn^{2+}-oxidizing activity. All

TABLE 1. Solubilization of the Mn^{2+}-oxidizing factor[a]

Fraction	Mn^{2+}-oxidizing activity (nmol of Mn^{2+} oxidized/ml · min)	Recovery (%)
Whole cells	5.0	100
Cell homogenate	8.0	160
Sup25k	7.1	142
Ppt25k[b]	0.4	8
Sup100k	6.2	124
Ppt100k[b]	0.4	8

[a] A 50-ml culture was used as the starting material. For experimental details, see Materials and Methods.
[b] The precipitates were resuspended in HEPES buffer to the original volume.

TABLE 2. Purification of the Mn^{2+}-oxidizing factor

Fraction	Vol (ml)	Total activity (nmol of Mn^{2+}/min)	Recovery (%)	Total protein (mg)	Sp act (activity/mg of protein)	Purification (fold)
Cell extract	200	9,489	100	2,465	3.9	1.0
$(NH_4)_2SO_4$ precipitate	75	15,861	167	204	78	20
Butyl-Toyopearl	5	2,764	29	7.2	382	100
Sephacryl S 300	5	217	2.3	0.45	482	125

purification procedures were performed at 4°C. The protein concentrations in the samples after each purification step were determined with the Bradford assay (5). The partially purified and concentrated Mn^{2+}-oxidizing preparation (Sephacryl fraction) was used for the characterization of the Mn^{2+}-oxidizing activity.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out on a 5 to 25% polyacrylamide gradient gel slab with the discontinuous solvent system of Davis (9). After electrophoresis, the gel was cut longitudinally into two parts. One part was stained with silver (15), and the other was used to detect Mn^{2+}-oxidizing activity as described by Boogerd and de Vrind (4). The latter gel slice was washed for 60 min with deionized water, which was refreshed every 15 min, and incubated in a solution of 1 mM $MnCl_2$ in 10 mM HEPES buffer. After 2 h of incubation, brown deposits of Mn oxide were detected.

Characterization of Mn^{2+}-oxidizing activity. The pH dependence of the Mn^{2+}-oxidizing activity was measured by diluting samples of the Sephacryl fraction 10 times in buffers with pHs varying from 5.5 to 8.2, using 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH for measurements at pH 7 or lower and 10 mM HEPES buffer for measurements at pH 7 or higher.

The temperature dependence of the Mn^{2+}-oxidizing activity was measured in 10-fold dilutions of the Sephacryl fraction in HEPES buffer (pH 7.4), which were preincubated at the relevant temperature for 10 min prior to the addition of $MnCl_2$.

The effects of different inhibitors and enzyme treatments on the Mn^{2+}-oxidizing activity of 10-fold dilutions of the Sephacryl fraction were measured by adding the various substances as concentrated solutions to final concentrations as indicated in Results. Enzyme treatments were performed at the optimal pH of each enzyme at 25°C for 30 min. Prior to the addition of $MnCl_2$, the pH was restored to 7.4.

RESULTS

Identification and characterization of the Mn^{2+}-oxidizing bacterium. The rod-shaped, gram-negative aerobic bacterium GB-1 (7) was identified as a *Pseudomonas* strain by the following characteristics. The bacterium oxidized glucose but did not ferment it, and it was oxidase positive. It was able to use citrate and to hydrolyze arginine. It oxidized xylose but not maltose or mannitol. It was not able to reduce nitrate and did not secrete DNase. The strain was preliminarily identified as *P. fluorescens* by partial 16S rRNA sequencing (6) (GenBank accession no. X87688).

In the early logarithmic growth phase, the cells were 4 to 7 μm long, 1 μm wide, and nonmotile. The cell size decreased to a length of 1 to 2 μm and a width of 0.7 μm in the late logarithmic and early stationary growth phases. In the stationary phase the cells had three polar flagellae, as detected by negative staining with uranyl acetate in TEM preparations.

TEM observations of cells which had been incubated in an $MnCl_2$ solution revealed the presence of electron-dense deposits surrounding the cells (Fig. 1, bottom). Electron-dense deposits were not formed by cells incubated in buffer without $MnCl_2$ (Fig. 1, top). The deposits contained large amounts of Mn as determined with X-ray microanalysis (data not shown) and most probably represent manganese oxide, because prefixation of the cells with the reducing agent glutaraldehyde resulted in dissolution of the deposits. The oxidizing activity was not released from the cells in the medium, as the spent culture medium of oxidizing cultures showed no activity. The Mn^{2+}-oxidizing factor apparently resides in the outer cell wall or cell cover of the bacteria.

Mn^{2+} oxidation by cells. The ability to catalyze the oxidation of Mn^{2+} depended on the growth phase of the bacteria. The first Mn^{2+}-oxidizing activity of cells was detected in cultures in the early stationary growth phase. The Mn^{2+} oxidation rate was directly proportional to the cell concentration in the OD_{660} range 0.2 to 1.0 and ranged from 1 to 9 nmol of Mn^{2+}/ml · min.

Effect of the oxygen concentration in the culture medium on Mn^{2+}-oxidizing activity. The bacteria were inoculated into 100 ml of culture medium in a 500-ml Erlenmeyer flask and grown with a shaking rate of 30, 60, or 85 strokes/min. The bacteria grew at identical rates under the different conditions, but the Mn^{2+}-oxidizing activity in the early stationary phases of the three cultures varied. An oxidation rate of about 4 nmol of Mn^{2+}/ml · min was obtained when cells were grown at 85 strokes/min, which was about four times higher than the activity of cells grown at 60 strokes/min. Cells had no activity when grown at 30 strokes/min. This result suggested a relationship between the Mn^{2+}-oxidizing activity and the oxygen concentration in the culture medium during growth of the bacteria. Figure 2A shows that the oxygen concentration (expressed as percent saturation at 20°C) in cultures in the late logarithmic phase was directly proportional to the shaking rate in the range of 50 to 100 strokes/min. In Fig. 2B the oxygen concentration

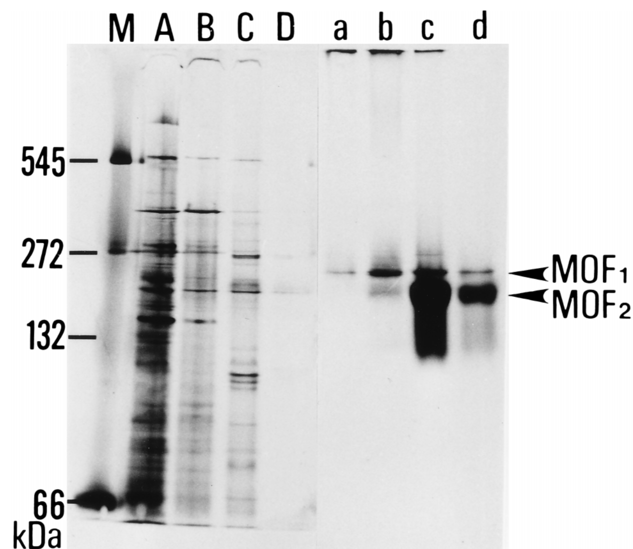

FIG. 3. Polyacrylamide gradient gel electrophoresis of Mn^{2+}-oxidizing samples after subsequent solubilization and purification steps (see also Table 2 and Fig. 4). Electrophoresis was carried out under nondenaturing conditions on a 5 to 25% gradient gel, and ca. 50 μg of protein was loaded in each lane. Lane M, molecular mass standards; lanes A and a, cell extract; lanes B and b, $(NH_4)_2SO_4$-precipitated proteins; lanes C and c, main Mn^{2+}-oxidizing fraction after butyl-Toyopearl chromatography; lanes D and d, Mn^{2+}-oxidizing peak fractions after Sephacryl S 300 chromatography. Lanes M, A, B, C, and D were stained with silver; lanes a, b, c, and d were stained for Mn^{2+}-oxidizing activity.

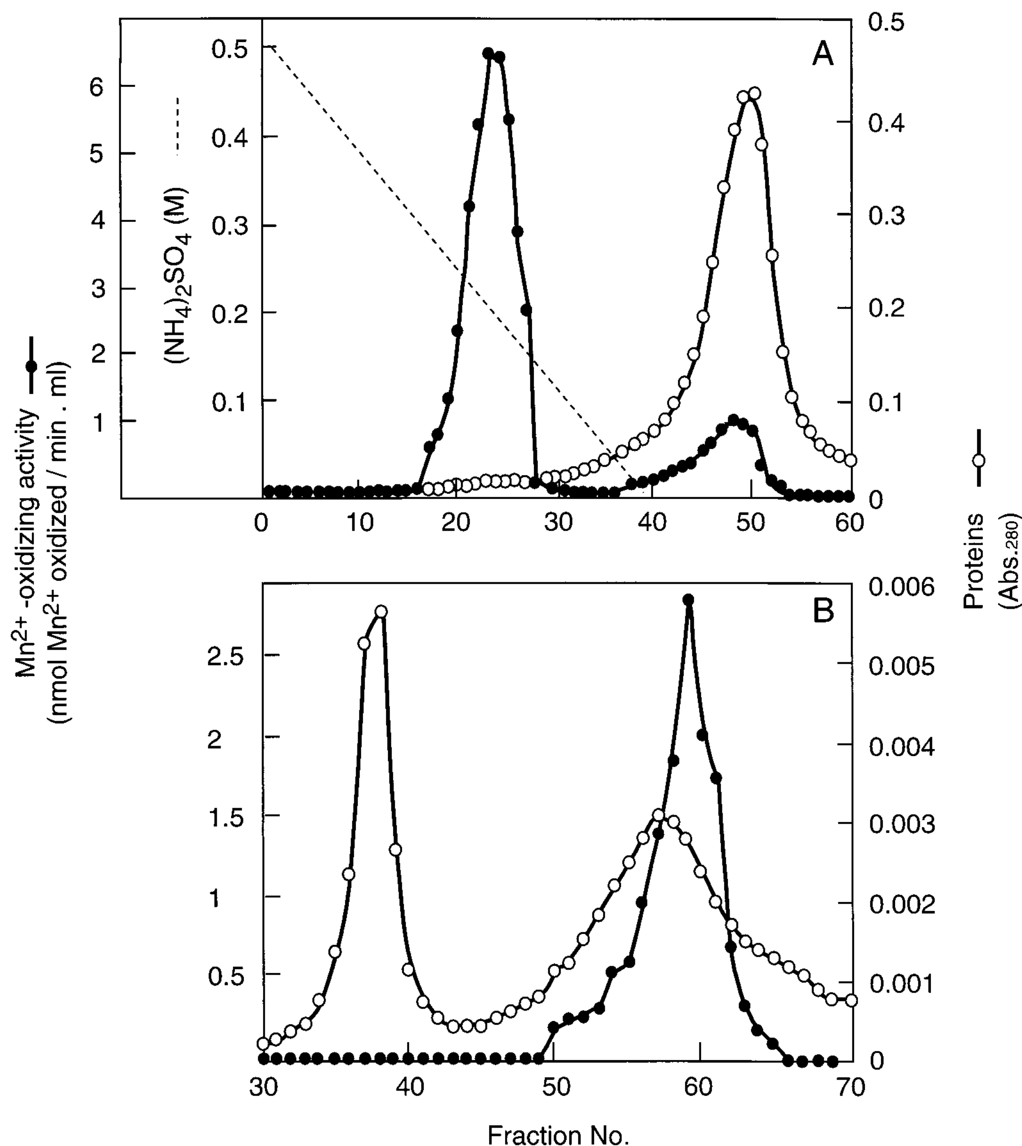

FIG. 4. Chromatography of Mn^{2+}-oxidizing samples on butyl-Toyopearl (A) and Sephacryl S 300 (B). The butyl-Toyopearl column was eluted with a gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in HEPES (– – –). Fractions 20 to 27 were pooled and, after precipitation and dissolution of the proteins, subjected to Sephacryl chromatography. Fractions 58 to 60 of the Sephacryl eluate were pooled and concentrated. The protein concentration in the eluate was indicated by the absorbance at 280 nm (○); Mn^{2+}-oxidizing activity was measured by the Leukoberbelin blue assay (●). Fractions of 5 ml were collected in each case. For further experimental details, see Materials and Methods.

in the culture medium in the late logarithmic phase (10 h after inoculation) is plotted against Mn^{2+}-oxidizing activity of whole cells in the early stationary phase (16 h after inoculation). No Mn^{2+}-oxidizing activity was seen when the oxygen concentration was below 14% saturation in the late logarithmic phase. The activity increased with the O_2 concentration from 15 to 26% saturation, followed by a decrease in the activity at higher oxygen concentrations. This result strongly suggests that the O_2 concentration in the culture medium at the late logarithmic phase has a profound effect on the amount of Mn^{2+}-oxidizing factor produced by the bacteria in the early stationary phase. The O_2 concentration in cultures during the early and mid-logarithmic growth phases appeared to be almost zero, even at high shaking rates. This was probably due to the high metabolic activity of the bacteria during these growth stages.

Solubilization of the Mn^{2+}-oxidizing activity. The Mn^{2+}-oxidizing activity could be solubilized by thorough disruption of the cells. The oxidizing activity of the cell extract was higher than that of the corresponding cell suspension, and only a minor part was recovered in the particulate fractions obtained by differential centrifugation of the cell homogenate (Table 1). Apparently part of the oxidizing activity was solubilized from cellular sites which are not directly in contact with the Mn^{2+}-containing medium. The protein content of the cell extract varied in different batches, from about 1.0 to 1.3 mg/ml. The rate of Mn^{2+} oxidation was proportional to the concentration of proteins in the cell extract within the range of 0 to 1 mg of protein/ml (not shown).

Purification of the Mn^{2+}-oxidizing components. Precipitation with $(NH_4)_2SO_4$, reversed-phase chromatography, and gel

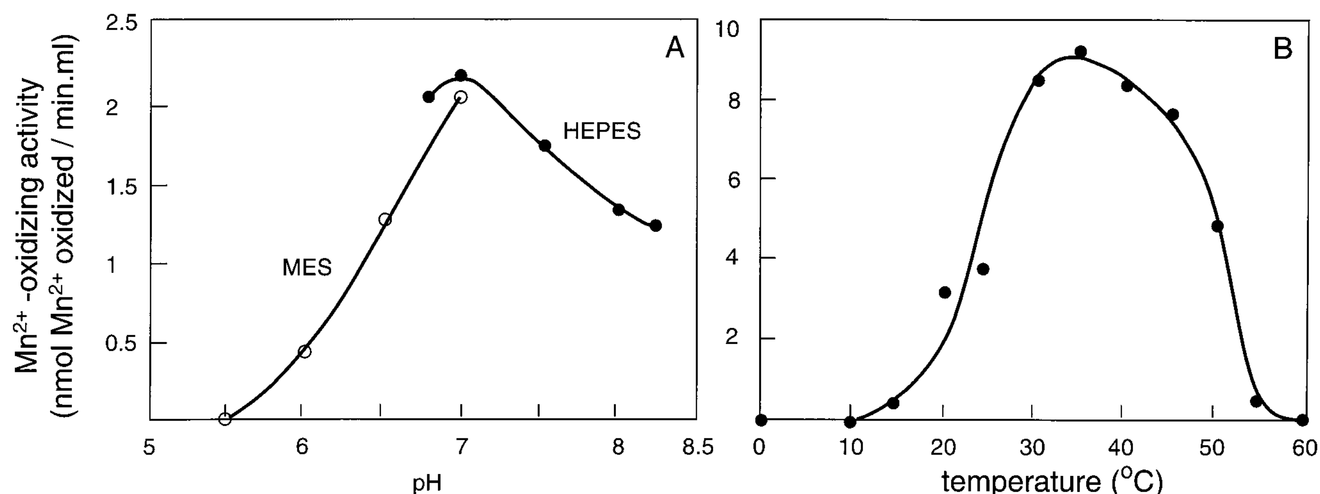

FIG. 5. Effects of pH (A) and temperature (B) on Mn^{2+}-oxidizing activity of the Sephacryl fraction (Table 2). The pH dependence was measured in 10 mM MES-NaOH (○) (pH 5.5 to 7.0) or in 10 mM HEPES-NaOH (●) (pH 6.8 to 8.2). The temperature dependence was measured for the Sephacryl fraction in HEPES buffer (pH 7.4); the samples were preincubated at each temperature for 10 min prior to the addition of $MnCl_2$.

filtration were applied to obtain a (partial) purification of the Mn^{2+}-oxidizing activity from the cell extract. After each separation procedure, the specific activity was determined (Table 2) and samples were examined by gradient gel electrophoresis (Fig. 3). The cell extract contained one major Mn^{2+}-oxidizing factor (MOF1), with an apparent molecular weight of 250,000, as determined by gel electrophoresis (Fig. 3, lanes A and a). The specific activity of Mn^{2+} oxidation increased by a factor of 20 after $(NH_4)_2SO_4$ precipitation of the proteins and their subsequent dissolution (Table 2). This fraction contained, in addition to MOF1, a second Mn^{2+}-oxidizing factor (MOF2) with an apparent molecular weight of 180,000 (Fig. 3, lanes B and b). Separation of the Mn^{2+}-oxidizing factors from the bulk of other proteins was established by reversed-phase chromatography (Fig. 4A; see also Table 2 and Fig. 3, lanes C and c). A further increase in specific activity was obtained by subsequent gel filtration of the pooled fractions from the main Mn^{2+}-oxidizing peak (Fig. 4B; see also Table 2 and Fig. 3, lanes D and d). In total, an approximately 125-fold purification was achieved. Both MOF1 and MOF2 were present in the sample after the last purification step. Whereas MOF1 was relatively most intensely stained for Mn^{2+}-oxidizing activity after $(NH_4)_2SO_4$ precipitation, MOF2 became more intensely stained after the chromatographic separations (Fig. 3, lanes b, c, and d).

Characterization of the Mn^{2+}-oxidizing factor. The characterization of the Mn^{2+}-oxidizing activity was performed with the partially purified preparation (Sephacryl fraction [Table 2]). The Mn^{2+}-oxidizing activity obeyed Michaelis-Menten kinetics in the range of 1 to 100 μM Mn^{2+} (not shown). The apparent K_m for Mn^{2+} was determined to be 10 μM.

The Mn^{2+}-oxidizing activity was pH dependent, with an optimum at a pH of approximately 7.0 (Fig. 5A). The activities in MES and HEPES buffers at around pH 7.0 were comparable, but the activity was strongly inhibited by Tris buffer (Table 3).

The Mn^{2+}-oxidizing activity was temperature dependent, with a maximal rate at 35°C (Fig. 5B). The activity was completely lost by heating the fraction at 60°C for 10 min. The activity was stable for at least 1 month upon freezing and storage at −20°C.

The effects of several enzyme inhibitors, complexing agents, and enzymatic treatments on the Mn^{2+}-oxidizing activity were tested (Table 3). NaN_3, an inhibitor of redox enzymes, completely inhibited Mn^{2+} oxidation at 1 mM. $HgCl_2$ completely inhibited the activity at 100 μM, and KCN did so at 10mM. The metal chelators EDTA and *o*-phenanthroline inhibited at 100 and 10 μM, respectively. Tris caused strong inhibition at 1 mM. Sodium dodecyl sulfate (SDS) at a low concentration (0.01%) stimulated the activity, but it was inhibitory at higher concentrations. Treatment of the Sephacryl fraction with lysozyme had no significant effect, whereas treatment with V8 protease or trypsin (at 10 or 100 μg/ml) increased the activity up to 50%. Treatment with lysylendopeptidase at 100 μg/ml resulted in a decrease in Mn^{2+}-oxidizing activity.

DISCUSSION

Manganese oxidation in *P. fluorescens* GB-1 is probably catalyzed by a protein which is part of the outer membrane or cell cover of the bacterium. Manganese oxides were never seen to be formed at periplasmic or intracellular sites, supporting the localization of the Mn^{2+}-oxidizing factor at the periphery of the cells. Several data indicate that the Mn^{2+}-oxidizing factor has a proteinaceous character. Like an enzyme, the Mn^{2+}-oxidizing factor has a pH optimum and a temperature optimum. Manganese oxidation obeys Michaelis-Menten kinetics and is inhibited by NaN_3, an inhibitor of redox enzymes, and by $HgCl_2$, which interacts with protein sulfhydryl groups. Inhibition of Mn^{2+} oxidation by KCN is probably due to complexation of Mn^{2+} ions by CN^- and not by interaction of KCN with the protein. It has been demonstrated that KCN, in contrast to NaN_3, affects the adsorption-desorption chemistry of Mn^{2+} by complexation of the ions (17). Likewise, complexation of Mn^{2+} by EDTA and probably by Tris results in inhibited Mn^{2+} oxidation. The metal chelator *o*-phenanthroline also inhibits Mn^{2+} oxidation but at relatively much lower concentrations than the other metal complexors. Complete inhibition is obtained at a concentration far below the concentration of Mn^{2+} in the medium. Consequently, the inhibitory effect of *o*-phenanthroline cannot be due to complexation of Mn^{2+}. This suggests that this substance interferes with the Mn^{2+}-oxidizing factor, possibly with metal centers in the protein. Metal center (copper-binding sites) were shown to occur also in proteins

TABLE 3. Effects of various inhibitors and enzyme treatments on Mn^{2+}-oxidizing activity

Inhibitor or enzyme	Concn	Relative activity (% of control)
NaN_3[a]	100 μM	65
	1 mM	0
$HgCl_2$[a]	1 μM	91
	10 μM	84
	100 μM	0
KCN[a]	100 μM	71
	1 mM	12
	10 mM	0
EDTA[a]	10 μM	100
	100 μM	0
	1 mM	0
o-Phenanthroline[a]	1 μM	70
	10 μM	0
	100 μM	0
Tris[a]	1 mM	11
	10 mM	0
SDS[a]	0.01%	134
	0.1%	0
	1.0%	0
Lysozyme[b]	10 μg/ml	94
	100 μg/ml	117
Staphylococcus aureus V8 protease[c]	10 μg/ml	151
	100 μg/ml	146
Trypsin[d]	10 μg/ml	138
	100 μg/ml	159
Lysylendopeptidase[d]	10 μg/ml	101
	100 μg/ml	38

[a] Activity of control, 3.2 nmol of Mn^{2+} oxidized/ml · min.
[b] Activity of control, 3.8 nmol of Mn^{2+} oxidized/ml · min.
[c] Activity of control, 3.3 nmol of Mn^{2+} oxidized/ml · min.
[d] Activity of control, 3.7 nmol of Mn^{2+} oxidized/ml · min.

involved in Mn^{2+} oxidation in *L. discophora* (7) and *Bacillus* strain SG1 (16).

The proteinaceous nature of the Mn^{2+}-oxidizing factor seems not to be in accordance with the stimulation of Mn^{2+} oxidation by SDS (at a low concentration) and by treatment with proteolytic enzymes like pronase and V8 protease. Only treatment with lysylendopeptidase appeared to reduce Mn^{2+}-oxidizing activity, but specific digestion products of this proteolytic treatment (or of any of the other digestions) were not found by gel electrophoresis (not shown). Apparently, none of the proteases used, or lysozyme, is able to specifically digest the Mn^{2+}-oxidizing factor. This relative insensitivity to or even stimulation by treatments of Mn^{2+}-oxidizing samples with SDS or proteases has also been reported for *L. discophora* (4) and *Bacillus* strain SG1 (10). The effect of the denaturing and digestive treatments on the Mn^{2+}-oxidizing activity of *P. fluorescens* GB-1 has been determined with partially purified cellular homogenates. The Mn^{2+}-oxidizing factors may be present as part of larger complexes, possibly of membraneous or cell wall origin. Treatments with SDS or proteases may expose active Mn^{2+}-oxidizing sites which were formerly less accessible for Mn^{2+}.

This report is the first to describe a partial purification of an Mn^{2+}-oxidizing factor with an increase in specific activity. The Mn^{2+}-oxidizing activity was solubilized from the cells with over 100% recovery, indicating that part of the Mn^{2+}-oxidizing activity had resided at intracellular or inaccessible cellular locations. Over 100% recovery of activity was also obtained after precipitation of proteins with $(NH_4)_2SO_4$. This procedure probably resulted in the removal of inhibiting substances. Two Mn^{2+}-oxidizing factors, MOF1 and MOF2, with apparent molecular weights of 250,000 and 180,000, respectively, could be distinguished by gel electrophoresis. Based on their Mn^{2+}-oxidizing activities, MOF1 was in excess over MOF2 in the cell extract, whereas the reverse was true for the purified samples. Possibly MOF2 was generated by degradation of a larger complex containing the Mn^{2+}-oxidizing factor, represented by MOF1. It cannot be excluded, however, that MOF1 and MOF2 represent two independent Mn^{2+}-oxidizing molecular species, of which MOF2 has been preferentially isolated.

Although progress has been made in the characterization and purification of the Mn^{2+}-oxidizing activity of *P. fluorescens* GB-1, the physiological role of the process is still unclear. The Mn^{2+}-oxidizing factor(s) appeared not to be produced before the early stationary phase of growth. Our results suggest that this production depends on the O_2 concentration in the culture prior to the stationary phase. The synthesis of the Mn^{2+}-oxidizing factor(s) may be regulated by sensors of the aerobic or anaerobic state of the bacterial culture. Possibly Mn^{2+} oxidation plays a role in the scavenging of toxic oxygen species (O_2^- and H_2O_2) produced during aerobic metabolism (16). A final decision about the cellular function of Mn^{2+} oxidation has to await the biochemical characterization of the factor(s) and the isolation of the genes involved in Mn^{2+} oxidation. This study lays the groundwork for the genetic analysis of the Mn^{2+}-oxidizing process in *P. fluorescens* GB-1.

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