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Electron transfer from acetate to the surface of MnO_2 particles by a marine bacterium

Henry L. Ehrlich

Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA

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SUMMARY

Intact cells of marine pseudomonad strain BIII 88, grown in the presence of added $MnSO_4$ (induced cells), reduced MnO_2 aerobically and anaerobically with acetate. They did not reduce limonite (FeOOH) with acetate. Spectrophotometric evidence of respiratory pigments in the cell envelope and inhibition of MnO_2 reduction by antimycin A and NQNO indicated that a respiratory process was involved. Stimulation of MnO_2 reduction by the oxidative phosphorylation uncouplers CCCP and 2,4-DNP indicated energy conservation during the reduction. Intact cells of strain BIII 88 grown in the absence of added manganese (non-induced cells) showed marginal MnO_2-reducing activity. Cell envelope fractions from induced cells prepared with a French press exhibited higher specific MnO_2-reducing activity on average than those prepared by sonication. Cell envelope fractions from induced cells contained more manganese than cell envelope fractions from non-induced cells. Recombined cell fractions from induced cells were more active than recombined cell fractions from non-induced cells. MnO_2 reducing activity was correlated with manganese content in cell envelope fractions. Cell envelope fractions from two cultures that do not reduce MnO_2 contained less manganese in their cell envelope fractions than similar fractions from non-induced strain BIII 88. Manganese in the cell envelope of strain BIII 88 appears to play a role in the transfer of reducing power from respiration on acetate across the cell envelope to the surface of MnO_2 particles.

INTRODUCTION

A number of bacteria are able to oxidize and/or reduce insoluble inorganic compounds like those found in minerals [9]. For example, the autotroph *Thiobacillus ferrooxidans* can oxidize chalcocite (Cu_2S) as its sole source of energy and reducing power, withdrawing electrons at the mineral surface from both cuprous copper and sulfide [7,22]. *Shewanella putrefaciens* (previously known as *Alteromonas putrefaciens*), *Geobacter metallireducens* (also known as strain GS-15), and *Bacillus polymyxa* strain D1 reduce manganese-oxides to Mn^{2+} in an anaerobic respiratory process [17,21,25,26]. *G. metallireducens* and *Shewanella putrefaciens* can use several different organic electron donors and *S. putrefaciens* also H_2 in this reductive process [17,18, 21]. *S. putrefaciens* and *B. polymyxa* are facultative organisms, which use MnO_2 as terminal electron acceptor only anaerobically. In contrast, some other unrelated freshwater and marine bacteria can reduce MnO_2 aerobically as well as anaerobically [5,8]. Indeed, in the case of *Bacillus* 29 and coccus 32, anaerobic conditions do not enhance MnO_2 reduction. MnO_2-reducing ability in *Bacillus* 29 is induced only aerobically [30]. Coccus 32 was not tested for this requirement for air.

MnO_2 (pyrolusite) is insoluble in water and in concentrated HNO_3. It is soluble only in concentrated HCl [31], forming manganese tetrachloride that disproportionates into manganese dichloride and chlorine on addition of water. This indicates that the dissociation of MnO_2 in water is so small that the concentration of reducible Mn^{4+} ions is too negligible to serve as electron acceptor in the respiratory metabolism of manganese reducing bacteria.

It remains to be explained how manganese reducing bacteria convey reducing power from the respiratory system in their cell envelope to MnO_2 with which they are in physical contact to reduce it. It may be a metal moiety in the cell envelope of an MnO_2 reducer, as occurs for instance in *Shewanella putrefaciens* [20], that serves as a shuttle for the reducing power. This would be analogous to the mechanism of electron withdrawal from insoluble metal sulfide by the gram-negative bacterium *Thiobacillus ferrooxidans* in which a polynuclear iron complex in the outer cell membrane has been implicated in the abstraction of reducing power from insoluble metal sulfides [12,15,16]. Alternative mechanisms to explain the electron transfer from metal sulfides have also been proposed [2,24,27,28,29].

The following study was aimed at characterizing the electron transport process in a marine pseudomonad when reducing insoluble MnO_2 with acetate under aerobic and anaerobic conditions and to seek evidence that a metal carrier may be involved in the transfer of reducing power across the cell envelope/MnO_2 particle interface.

An understanding of the mechanism of transfer of reducing power across the interface between the bacterial

Correspondence to: Prof. H.L. Ehrlich, Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA.

cell envelope and the surface of an oxidizable or reducible mineral particle has great significance for bioleaching of ores by direct attack.

MATERIALS AND METHODS

Cultures

Marine pseudomonad strain BIII 88 is a gram-negative rod isolated from deep-sea sediment associated with a ferromanganese nodule deposit. The sediment was collected from a site in the Central Pacific Ocean. The culture grows in media prepared with seawater or 3% NaCl but not with fresh water. Preliminary tests indicate an affinity to the genus *Pseudomonas*. Stock cultures of the strain were carried on nutrient agar made up in natural seawater and incubated at 15 °C.

Manganese oxidizers *Oceanospirillum* strains BIII 45 and BIII 82 [1] and marine bacterial strain BIII 77 were used in comparative tests. Intact cells of these three strains did not reduce MnO_2 in prior tests (Ehrlich, unpublished). They were grown in the same medium as pseudomonad BIII 88.

Media

Cell crops of strain BIII 88 for examining the difference spectrum of a cell envelope preparation, and for studying the effect on MnO_2-reduction of electron transport inhibition, uncoupling of energy conservation, and inhibition by *o*-phenanthroline and 2,2′-dipyridyl were grown on slants of nutrient agar (Difco) in Roux bottles prepared in natural seawater containing 4.8 mM $MnSO_4 \cdot H_2O$. Incubation was for 14–16 h at 15 °C. The cells were harvested by removal from the agar surface with 0.5 M NaCl and washed by centrifugation at 12 000 × *g* and 4 °C in 0.5 M NaCl until 1.0 ml of the washings showed an absence of manganese detectable by the persulfate oxidation assay (usually four washings). The washed cells were then resuspended in 5 ml 0.5 M NaCl.

Cell crops for tests on cell fractions were grown in liquid medium in shake culture at 17–20 °C for 14 h except where otherwise specified. The medium was nutrient broth (Difco) prepared in synthetic seawater (g per liter of distilled water: NaCl, 24.3; $MgCl_2 \cdot 6H_2O$, 11; $Na_2SO_4 \cdot 10H_2O$, 4.1; $CaCl_2 \cdot 2H_2O$, 1.51; KCl, 0.7). For producing induced cells, the nutrient broth also contained 4.8 mM $MnSO_4$. Cells were harvested and washed in 0.5 M NaCl by centrifugation, using the same procedure as for the cells from Roux bottle slants. Cells grown in liquid medium exhibited the same MnO_2-reducing activity in resting cell suspension as cells grown on seawater–nutrient agar in Roux bottles.

Preparation of crude cell extract and cell fractions

Crude cell extract and some cell fractions were prepared by sonicating washed cells in a crushed ice bath for 2–3 min with a Bronwill Biosonic sonicator at a power setting of 68 W cm^{-2}. The sonicate was then centrifuged at 11 000 × *g* at 4 °C for 10 min in a Sorvall RC-2B centrifuge to remove unbroken cells. The supernatant fluid after decantation constituted crude extract. For fractionation, the crude extract was centrifuged for 20 min at 43 000 × *g* at 4 °C. The resultant pellet constituted the cell envelope fraction, which, after decantation of the supernatant fluid, was resuspended in 0.5 M NaCl. The supernatant phase was centrifuged at 204 000 × *g* for 1 h at 4 °C in a Ti60 anglehead rotor in a Beckman ultracentrifuge (model L8-55). The resultant pellet was discarded. The supernatant fluid represented the soluble fraction.

Other cell envelope fractions were prepared by passing cells twice in succession through a French pressure cell chilled to 5 °C and pressurized to 357 bar (5000 lbs in^{-2}). The resultant extract was then fractionated by the same successive centrifugations as the sonicates.

MnO_2 reduction

MnO_2 reduction by intact cells, crude extract, or cell fractions was tested by overlaying 0.1 g of washed, reagent grade MnO_2 (Baker analyzed) with 7.2 ml of a reaction mixture containing 0.5 mM sodium acetate in 0.48 M NaCl. Experimental reaction mixtures contained a measured quantity of intact cells, crude cell extract, or cell fractions. Controls included setups without cells or cell fractions to determine abiotic manganese release from MnO_2, and cells or single and recombined cell fractions without MnO_2 to determine manganese release from cells or cell fractions during incubation of the reaction mixtures. The inhibitors antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (2,4-DNP), *o*-phenanthroline, and 2,2′-dipyridyl were tested singly at specified concentrations.

Quantification of cells was by cell number or by total protein in non-centrifuged crude sonicate from a measured sample of cell suspension. Quantification of cell extract or cell fractions was by protein determination. Aerobic incubations of reaction mixtures was in 50-ml Erlenmeyer flasks whereas anaerobic incubation was in Thunberg tubes (160 × 18 mm) in which the air was replaced with oxygen-free nitrogen (Union Carbide, Linde Division) in 5 successive evacuations. The incubation for all MnO_2 reduction experiments was in stationary mode at 15 °C for 4 h. The pH in all reaction mixtures remained at 7.0 throughout their incubation. Aerobic reaction conditions, except tests with cell envelope fractions and supernatant phases incubated without added MnO_2, were run in duplicate. Aerobic tests were generally tested with at least two separate cell preparations. Anaerobic tests were run singly but tested with at least two separate cell preparations.

The extent of MnO_2 reduction was measured in terms of solubilized manganese. For the determination, the contents of all flasks or Thunberg tubes were acidified at the end of 4 h with 0.05 ml of 10 N H_2SO_4, mixed and allowed to stand for 10 min. A 3-ml aliquot was then removed and centrifuged. One milliliter of the supernatant fluid was assayed in duplicate for dissolved manganese.

Limonite reduction

Tests for reduction of 0.1-g amounts of ground limonite ($FeOOH \cdot nH_2O$) (Ward's, Rochester, NY) with acetate by

intact cells of pseudomonad BIII 88 were conducted in the same way as for MnO_2. The particle size of the limonite was in the range of 63–125 μm.

Determination of Mn content of cell envelope fractions

A measured amount of cell envelope fraction prepared by sonication or with a French press as described above was transferred to a 10-ml Kjeldahl flask and wet-ashed by digestion with 1 ml of concentrated sulfuric acid. The resultant digest was analyzed for manganese by atomic absorption spectrophotometry. The amount of cell envelope fraction was quantified in terms of its protein content.

Mn assays

Two methods were used to measure manganese released from MnO_2 in MnO_2 reduction. One method was the persulfate oxidation test described by Ehrlich [6], except that the analysate consisting of sample, diluent, special reagent [6] and 0.1 g of potassium persulfate was boiled for 12 min with five further additions of 0.1 g of potassium persulfate at 2-min intervals. This method was used for all experiments with intact cells and crude cell extracts. With this assay, no manganese was detected in 1-ml samples from controls containing MnO_2 but no cells or crude extract, or in controls with cells or crude extract but lacking MnO_2. Using this manganese assay, MnO_2-reducing activity was based on the amount of dissolved Mn measured in the supernatant phase after centrifugation of an aliquot from the acidified reaction mixture.

The second method whereby manganese released in MnO_2 reduction tests was measured, was by atomic absorption in an air/acetylene flame with a Perkin Elmer model 1100B instrument (sensitivity for Mn, 0.052 mg L^{-1}). This method was used exclusively for measuring the MnO_2-reducing activity of cell fractions because the organic matter, which could not be removed by centrifugation as when intact cells or crude extract were tested, interfered with the persulfate assay.

The atomic absorption assay is more sensitive than the persulfate assay. It detected 5.0 nmol Mn ml^{-1} spontaneously released from MnO_2 in control flasks whereas the persulfate oxidation assay did not. Therefore correction had to be made in this assay for manganese in reaction mixtures that did not originate from enzymatic reduction of MnO_2. For active preparations, the corrections involved the subtraction of dissolved manganese derived from the cell envelope fraction and the soluble fraction incubated without MnO_2. For inactive preparations, dissolved Mn found in controls without any cell fraction but with MnO_2 was also subtracted. The latter correction was not made for active cells or cell fractions, because in many tests with individual cell envelope fractions or soluble fractions, subtraction of this control resulted in a large negative value. It was inferred that active cells and cell fractions removed the manganese that was abiotically released from MnO_2. A portion of the abiotically released manganese may have been colloidal MnO_2.

Although tests for MnO_2-reducing activity were always run with individual supernatant and cell envelope fractions as well as recombined fractions, all activities reported in this paper refer only to recombined fractions (1:1 by volume). Cell envelope fractions alone were either inactive or showed lower activity by themselves than when mixed with supernatant fraction. Some supernatant fractions exhibited MnO_2-reducing activity without added cell envelope fraction, but this activity was less than in corresponding recombined fractions and was probably due to incomplete separation of small membrane vesicles in ultracentrifugation.

Iron assays

Solubilized iron in the limonite reduction tests was determined in centrifuged supernatant phases using atomic absorption spectrophotometry with an air/acetylene flame (sensitivity for Fe, 0.1 mg L^{-1}).

Difference spectrum of cell envelope fraction from strain BIII 88

A cell envelope fraction was prepared from a sonicate of pseudomonad BIII 88 by differential centrifugation as described above. However, the cell envelope fraction was collected at 32 500 × *g* instead of 43 000 × *g* and resuspended in 7 ml of 0.46 M NaCl containing 0.01 potassium phosphate at pH 7.5. The cell envelope concentration was quantified by protein determinations. A dithionite-reduced-minus-oxidized difference spectrum was generated in a Shimadzu-Bausch and Lomb UV 200, split-beam, scanning spectrophotometer equipped with a Houston Instrument Omnigraph X–Y recorder.

Protein determination

Protein was determined by the Lowry method [19]. In the case of protein determination on intact cells, a measured volume of the suspension was first sonicated as previously described and an aliquot of the sonicate assayed without prior centrifugation. Bovine serum albumin served as standard.

RESULTS

Aerobic and anaerobic reduction of MnO_2

Induced resting cells of pseudomonad strain BIII 88 reduced MnO_2 aerobically and anaerobically with acetate as electron donor. Involvement of the electron transport system was inferred from the effect of electron transport inhibitors HQNO and antimycin A (Table 1) on manganese solubilization. It is noteworthy that under aerobic conditions, the inhibitors stimulated MnO_2 reduction slightly at the concentration used while under anaerobic conditions they inhibited it. The stimulation is best explained on the basis of a branched electron transport pathway to oxygen and MnO_2 in which the branch to oxygen is more sensitive to the inhibitors than the branch to MnO_2. The aerobic and anaerobic paths to MnO_2 may not be identical. When glucose replaced acetate, MnO_2 reduction by strain BIII 88 was inhibited aerobically in the presence of antimycin A and NQNO.

Although the specific activity in the absence of any inhibitor under anaerobic conditions was smaller than under

TABLE 1

Inhibition of electron transport from acetate to MnO_2 in cell extracts from induced strain BIII 88

Condition	Substrate[b]	Inhibitor[c]	Mn^{2+} released from MnO_2[a] (nmol h^{-1} mg^{-1} protein)	
			Expt. 1	Expt. 2
Aerobic	acetate	none	74.6	72.1
	acetate	antimycin A	81.6	89.3
	acetate	HQNO	79.6	87.1
Anaerobic	acetate	none	52.3	50.7
	acetate	antimycin A	32.7	32.1
	acetate	HQNO	40.9	36.7

[a] Mn determination by persulfate oxidation method.
[b] Acetate concn. 5 mM.
[c] Antimycin A concn. 10.8 μM; 2-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO) concn. 54 μM.

TABLE 2

Effect of (CCCP) on MnO_2 reduction with acetate by intact, induced cells of strain BIII 88

Experimental condition	CCCP concn. (nM)	Mn solubilized from MnO_2 (nmol h^{-1} by 10^9 cells)[a]		Percent stimulation
		−CCCP	+CCCP[b,c]	
Aerobic[d]	340	9.9	18.0	82
	340	4.4	7.3	66
	340	3.7	6.5	76
		mean 6.0±3.4	10.6±6.4	75±8.1
Anaerobic[d]	340	6.6	12.3	86
	340	8.2	15.0	83
	340	5.5	8.3	51
	340	4.9	11.0	124
	340	4.7	7.9	68
		mean 5.98±1.4	10.9±2.9	82.4±27.1
	170	6.5	8.2	26
	170	6.2	7.8	26
		mean 6.35±0.2	8.0±0.3	26±0
	35	9.9	12.3	24
	35	17.2	21.1	23
	35	7.5	10.2	36
		mean 11.5±5.1	14.5±5.8	27.7±7.2

[a] Mn determined by persulfate oxidation method.
[b] No measurable reaction of CCCP with MnO_2 at any CCCP concentration.
[c] No stimulation of MnO_2-reducing activity of strain BIII 88 by ethanol, the solvent for CCCP, when at 0.25 M concentration in the reaction mixture. MnO_2 was not reduced by strain BIII 88 aerobically or anaerobically with 0.25 M ethanol as sole electron donor.
[d] Acetate concn. 0.5 mM.

aerobic conditions, this difference may be an artifact. It could have been due to the difference in the amount of surface exposed by MnO_2 in the respective reaction vessels. In the Erlenmeyer flasks, the MnO_2 was spread out over the bottom of the flasks (45 mm diameter), whereas in the tubes it was confined to a button on the concave bottom of the tubes (18 mm diameter).

A dithionite-reduced-minus-oxidized difference spectrum of cell envelope fraction at a protein concentration of 0.6 mg ml^{-1} of strain BIII 88 revealed alpha-peaks at 563 and 552 nm and beta peaks at 533 and 524 nm, indicating the presence of *b*- and *c*-type cytochromes.

Anaerobic reduction of limonite

Intact resting cells of strain BIII 88 (1.7×10^9 ml^{-1}) did not reduce limonite with acetate in 4 h of incubation. They produced no measurable net increase in soluble iron as measured by atomic absorption spectrophotometry.

Energy conservation

Aerobic and anaerobic tests for MnO_2 reduction with acetate by induced resting cells of strain BIII 88 in the presence of the oxidative phosphorylation uncouplers CCCP and 2,4-DNP showed that the organism conserves energy in the process. This was indicated by the stimulation of manganese solubilization at appropriate concentrations of the inhibitors (Tables 2 and 3). The uncoupling of oxidative phosphorylation by compounds like CCCP and 2,4-DNP in respiration is often manifested by enhancement in oxygen consumption in an aerobic process [13] but can also be demonstrated by enhancement in substrate consumption [11], and as a corollary, to alternate-electron-acceptor reduction.

Effect of metal chelators

Induced resting cells of strain BIII 88 were incubated aerobically and anaerobically in the presence and absence of appropriate concentrations of 2,2′-dipyridyl and *o*-phenanthroline, effective chelators of iron and known to interfere with electron transport functions of iron [14,23]. The results in Table 4 indicate that while 2,2′-dipyridyl inhibited MnO_2 reduction at 2.2 mM, *o*-phenanthroline did not inhibit it significantly at 1.9 mM. This rules out a role for iron in a polynuclear complex as electron carrier in the cell envelope, as is found in *T. ferrooxidans*. It is assumed that the iron in the polynuclear complex is chelatable by the dipyridyl and *o*-phenanthroline reagents. The affinity constants (log K) of 2,2′-dipyridyl for Fe^{2+} range from 17.16 to 17.45 and of *o*-phenanthroline range from 20.48 to 21.3 [3]. The corresponding affinity constants for Mn^{2+} range from 2.6 to 5.9 (2,2′-dipyridyl) and 3.8 to 4.13 (*o*-phenanthroline). If chelatable iron had been involved in the reduction of MnO_2, both chelators should have had an inhibitory effect. The differential inhibitory effect cannot be interpreted in terms of reaction with manganese as a carrier in the envelope because of its low affinity constant for both ligands. At this time no explanation can be offered for the effective

TABLE 3

Effect of 2,4-dinitrophenol (2,4-DNP) on MnO_2 reduction with acetate by intact, induced cells of strain BIII 88

Experimental condition[a]	2,4-DNP concn. (μM)	Mn solubilized[b] (nmol h^{-1} per 10^9 cells)		Percent stimulation[c]
		−2,4-DNP	+2,4-DNP	
Aerobic	611	6.8	4.2	−38
	306	7.3	6.4	−12
	153	15.8	13.9	−12
	83	11.4	13.2	16
	83	9.9	12.6	27
Anaerobic	83	8.0	8.8	10
	41.5	6.4	10.6	66
	41.5	9.4	13.1	39
	14	9.9	9.9	0
	14	8.0	8.0	0

[a] Initial acetate concentration was 0.5 mM.
[b] Mn assays by persulfate oxidation method.
[c] $[Mn_{+DNP} - Mn_{-DNP}/Mn_{-DNP}] \times 100$. Negative values indicate inhibition.

TABLE 4

2,2′-Dipyridyl and *o*-phenanthroline inhibition of MnO_2 reduction by intact cells of induced strain BIII 88

Experimental condition	Inhibitor	Inhibitor concentration (mM)	% inhibition[a]
Aerobic	2,2′-dipyridyl	2.2	100
	2,2′-dipyridyl	2.2	100
	o-phenanthroline	1.9	6
	o-phenanthroline	3.9	1
Anaerobic	2,2′-dipyridyl	2.2	100
	2.2′-dipyridyl	2.2	100
	o-phenanthroline	1.9	17
	o-phenanthroline	1.9	0[b]

[a] All Mn assays by persulfate oxidation method.
[b] Below detection limit of persulfate assay.

inhibition of MnO_2 reduction by 2,2′-dipyridyl but not *o*-phenanthroline.

Cell-free preparations

The specific activities of eight pairs of cell-free preparations from strain BIII 88 were compared. One of each pair of preparations was obtained by sonication and the other by French press cell disruption. The manganese solubilized by them from MnO_2 was measured by atomic absorption. On average, the specific activities of preparations from French press disruption were more active than corresponding sonicate preparations (127 ± 176 versus 53 ± 101 nmol Mn released from MnO_2 h^{-1} mg^{-1}). The large standard errors reflect the variability in activity between preparations of each kind. Although both sonication and French press cell disruption have been reported to yield mainly everted envelopes (inside-out orientation) [4], these results indicate that the inside-out orientation was more prevalent in the sonicate preparations than in the French press preparations, on the assumption that a right-side-out orientation is required for MnO_2 reduction. A right-side-out orientation should be the preferred orientation for MnO_2 reduction because it is the orientation in intact cells when they reduce MnO_2 while in contact with it.

Mn in cell envelope fractions from induced and uninduced cells

With intact resting cells of strain BIII 88, the persulfate oxidation assay detected MnO_2 reduction only when the cells were induced. When MnO_2 reduction by intact cells was assayed by the more sensitive atomic absorption method, induced resting cells exhibited a specific MnO_2-reducing activity of 16 ± 9 nmol Mn h^{-1} mg^{-1} cell protein whereas non-induced resting cells exhibited specific MnO_2-reducing activity of 2 ± 0.6 nmol Mn h^{-1} mg^{-1}. The added Mn^{2+} in amended seawater-nutrient broth for producing induced cells of strain BIII 88 stimulated their growth (Fig. 1).

Manganese was taken up by the cells during growth in manganese-supplemented medium. A portion of it was released when the cells were harvested and washed in 0.48 M NaCl by centrifugation. As Table 5 shows, more manganese was retained by the cell envelope fraction of induced strain BIII 88 after washing with 0.48 M NaCl at

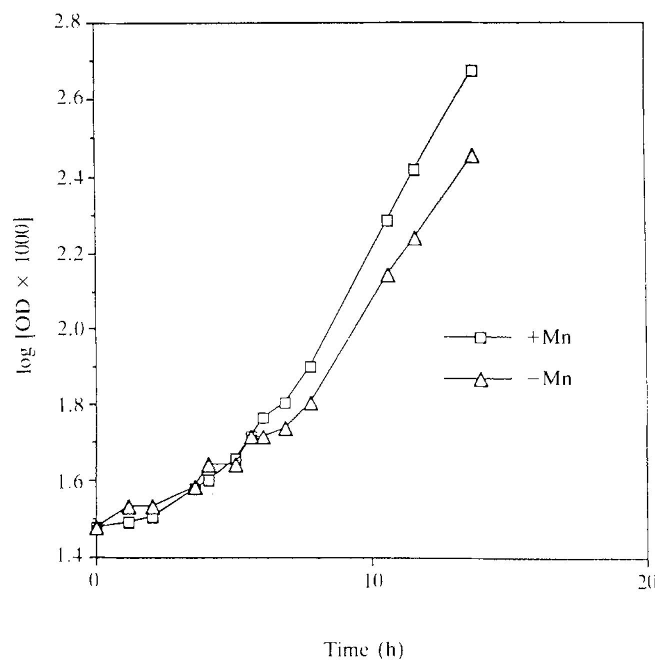


Fig. 1. Growth curves of marine pseudomonad BIII 88 in shake culture at 20 °C. (-△-) Growth in unamended synthetic seawater–nutrient broth; (-□-) Growth in synthetic seawater–nutrient broth amended with 4.8 mM $MnSO_4$. Counts of colony-forming units by surface-spread plating in triplicate on unamended synthetic seawater–nutrient agar were $8.0 \times 10^7 \pm 9.0 \times 10^6$ for curve (a) and $1.7 \times 10^8 \pm 1.6 \times 10^7$ for curve (b) after 10.65 h of incubation.

5 °C than at room temperature. This indicates that manganese release from the cells may be temperature-dependent and therefore affected by metabolic activity.

The data in Table 5 also show that less Mn was found in cell envelope fractions from induced cells of strain BIII 88 grown for 10 h than from cells grown for 14 h.

On average, cell envelope fractions from non-induced, 14 h-old cells of strain BIII 88 contained a smaller but still significant amount of manganese than induced cells. With the atomic absorption assay method, these cell envelopes exhibited MnO_2-reducing activity when recombined with the soluble fraction, but it was lower when compared to that of cell envelope preparations from 14 h-induced cells. A strong correlation ($r^2 = 0.956$) was found between Mn content of the cell envelope fractions from these non-induced cells and MnO_2-reducing activity. Cell envelope fractions from induced cells contained more manganese than the fractions from non-induced cells. If the activity measurement for the 14 h-induced, chilled envelope preparation is disregarded, the activity of the remaining three fractions from the induced cells correlates with their respective Mn content ($r^2 = 0.959$). When the data for all induced preparations except for the 14 h-chilled one, and all non-induced envelope fractions were combined, activity and Mn content correlated significantly ($r^2 = 0.813$). A portion of the Mn in the cell envelope preparation of the chilled 14 h-induced cells must not have participated directly in MnO_2 reduction in the corresponding activity test. A small portion of the Mn in the remaining three envelope preparations from the induced cells also may not have participated directly in MnO_2 reduction.

To determine whether other bacterial cultures in which MnO_2-reducing activity cannot be induced contain lesser amounts of manganese in cell envelope fractions prepared by cell rupture in the French press than non-induced cells of strain BIII 88, two Mn(II)-oxidizing strains, *Oceanospirillum* BIII 45 and BIII 82 [1], grown for 21 h in unamended seawater–nutrient broth, were tested. As Table 5 shows, the cell envelope fractions contained about an order of magnitude less Mn than non-induced cells of strain BIII 88 and exhibited marginal MnO_2-reducing activity.

The cell envelope fraction of from 20 h-old cells of marine strain BIII 77, a culture whose intact cells neither oxidize Mn(II) nor reduce MnO_2 when tested by the persulfate assay method, contained about as much manganese when grown in unamended seawater–nutrient broth as the cell envelope fraction from non-induced strain BIII 88. Recombined fractions exhibited MnO_2-reducing activity at the same level as preparations from non-induced strain BIII 88.

The MnO_2-reducing activity exhibited by recombined fractions from non-induced cells of strain BIII 88 was mostly if not entirely enzymatic. $HgCl_2$ (1.4 mM) inhibited a French-press preparation 50% and a sonicate preparation 68%. Boiling for 15 min inhibited a French-press preparation 51%

TABLE 5

Mn concentration in cell envelope fraction from induced and non-induced cells of several marine bacterial strains

Strain	Processing condition	Mn concn. in cell envelope fraction[a] (nmol Mn mg^{-1} protein)		Mn(IV)-reducing activity[b] (nmol Mn(II) mg^{-1} h^{-1})
		Induced[c]	Non-induced[d]	
BIII 88	10-h cells, chilled	655		49
	10-h cells, not chilled	367		50
	14-h cells, chilled	7010		107
	14-h cells, not chilled	1750		209
	14-h cells, chilled		535	50
	14-h cells, chilled		227	37
	14-h cells, chilled		1320	80
	14-h cells, chilled		545	46
	14-h cells, chilled		290	35
	14-h cells, chilled		390	34
BIII 77[e]	20-h cells, chilled		274	78
BIII 45[e]	21-h cells, chilled		38	9
BIII 82[e]	21-h cells, chilled		54	15

[a] Cell envelope fractions prepared by French press treatment. All activities represent those of recombined cell fractions. The Mn content of each cell envelope preparation was determined on a portion not used in the activity test.

[b] Manganese measured by atomic absorption method.

[c] Cells grown in synthetic seawater–nutrient broth containing 4.8 mM $MnSO_4$.

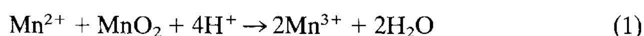
[d] Cells grown in synthetic seawater–nutrient broth without added $MnSO_4$.

[e] Intact cells of this strain do not reduce MnO_2 whether grown in presence or absence of added $MnSO_4$.

and a sonicate preparation 37%. Boiling another sonicate preparation for 25 min inhibited it 57%. These results should be considered apparent inhibitions because it was not determined to what extent, if any, residual activity was due to chemical reduction of MnO_2.

DISCUSSION

These findings show that pseudomonad BIII 88 is able to reduce MnO_2 aerobically and anaerobically with acetate as electron donor. In the cells, the reducing power from acetate appears to be fed into their electron transport system. The significant correlation between manganese content of cell envelopes and their MnO_2-reducing activity suggests that manganese in the cell envelope may be directly involved in conveying the reducing power across the cell envelope/ MnO_2 particle interface. As Mn^{2+}, it may be a reactant in the disproportionation,



$$Mn^{2+} + MnO_2 + 4H^+ \rightarrow 2Mn^{3+} + 2H_2O \qquad (1)$$

The resultant Mn^{3+} may then become the acceptor for the reducing power from acetate oxidation in a reaction that can be formulated in a half-reaction as

$$Mn^{3+} + e^- \rightarrow Mn^{2+} \qquad (2)$$

The manganese in the cell envelope may thus be an electron shuttle.

Anaerobically, all reducing power from acetate oxidation must be shunted to the manganese shuttle in the proposed model. Aerobically, only a portion of the reducing power is shunted to the manganese shuttle, the rest to oxygen. Although not determined in these experiments, more acetate should be consumed by the cells aerobically than anaerobically since resting cells reduced similar amounts of MnO_2 under both conditions.

The present experiments leave unresolved in what part of the cell envelope the bound manganese occurs because the envelopes included both outer and inner membrane and periplasm. If it occurs only in the outer membrane, a periplasmic shuttle would be required to transfer reducing power from the electron transport system in the plasma membrane to the outer membrane.

The induction of MnO_2-reducing activity in strain BIII 88 may involve a modification of the cell envelope resulting in enhanced manganese incorporation from the medium. The slower rate of MnO_2 reduction exhibited by cell-free preparations from non-induced cells compared to induced cells correlated with the amount of bound manganese found in the respective cell envelopes. The presence of Mn^{2+} in the cell envelope fraction of non-induced cells of strain BIII 88, though less than in induced cells, may explain why non-induced, growing cells of cultures like BIII 88 can initiate MnO_2 reduction in the absence of added Mn^{2+} but in the presence of MnO_2 in the medium, as previously reported [30].

The use of manganese as electron carrier from the cell envelope to the surface of MnO_2 particles may explain why pseudomonad strain BIII 88 can reduce MnO_2 aerobically as well as anaerobically while *Shewanella putrefaciens*, *Geobacter metallireducens*, and *Bacillus polymyxa* D1 can do so only anaerobically. These latter organisms may use Fe as a shuttle in place of manganese. Since Fe^{2+} is very susceptible to autoxidation at neutral pH whereas Mn^{2+} is not, organisms using iron in place of manganese as electron shuttle could do so only anaerobically. Myers and Myers [20] have reported that *S. putrefaciens* contains significant amounts of *c*-type cytochrome in its outer membrane when grown anaerobically but not aerobically. The *c*-type cytochrome in the outer membrane of the anaerobically grown cells could represent this iron or it could be a carrier for reduction of ferric iron in a polynuclear complex like that found in the outer membrane of *T. ferrooxidans*.

Thermodynamically iron can be a more efficient electron shuttle to MnO_2 than manganese. Calculations show that with iron as shuttle, the standard free energy potentially available to the cells per mol of MnO_2 reduced by acetate is −223.2 kJ (−53.4 kcal). With manganese as electron shuttle, the standard free energy available to the cells per mol of Mn^{3+} reduced to Mn^{2+} is −138.6 kJ (−33.2 kcal). The energy from the disproportionation of MnO_2 is assumed not to be available to the cells. For a further discussion of a model to explain MnO_2 reduction by strain BIII 88 see the discussion by Ehrlich [10].

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