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Enzymatic Iron Oxidation by *Leptothrix discophora*: Identification of an Iron-Oxidizing Protein

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An iron-oxidizing factor was identified in the spent culture medium of the iron- and manganese-oxidizing bacterial strain *Leptothrix discophora* SS-1. It appeared to be a protein, with an apparent molecular weight of approximately 150,000. Its activity could be demonstrated after fractionation of the spent medium by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A spontaneous mutant of *L. discophora* SS-1 was isolated which excreted neither manganese- nor iron-oxidizing activity, whereas excretion of other proteins seemed to be unaffected. Although the excretion of both metal-oxidizing factors was probably linked, the difference in other properties suggests that manganese and iron oxidation represent two different pathways. With a dot-blot assay, it was established that different bacterial species have different metal-oxidizing capacities. Whereas *L. discophora* oxidized both iron and manganese, *Sphaerotilus natans* oxidized only iron and two *Pseudomonas* spp. oxidized only manganese.

Microorganisms can play a dominant role in the global and local cycles of the elements. They may therefore be considered geological forces (7, 15). Iron and manganese are among the most abundant metals in the Earth's crust (7, 13). Their cycling is strongly influenced by their redox state. Iron is commonly found as Fe(II) and Fe(III) and manganese as Mn(II) and Mn(IV) and to a lesser extent as Mn(III). The reduced states represent the soluble and mobile forms, whereas oxidation results in the precipitation of highly insoluble oxides and oxyhydroxides (13). Conversion between the redox states is often catalyzed by bacteria. The organisms involved in these conversions can therefore be considered important geomicrobial agents (7).

Leptothrix discophora is a sheath-forming bacterial species which is characterized by the precipitation of iron and manganese oxides on its sheaths. The manganese oxide is thought to be generated by an enzymatic process in which a sheath component serves as the catalyst (8). Strain SS-1 was first described by Adams and Ghiorse (1, 2). Upon subcultivation in the laboratory, it lost its ability to form a structured sheath. It retained its manganese-oxidizing ability, and during growth, a manganese-oxidizing factor is excreted into the medium (3, 4). This factor may be identical to the manganese-oxidizing sheath component of sheath-forming cells.

In contrast to manganese oxidation, the precipitation of iron oxides on the sheaths of bacteria in nature is often thought to be a nonspecific process, resulting from the binding of reduced iron species to anionic groups on the sheath surface, followed by nonbiological oxidation to iron oxides (7, 8).

In a previous study, we demonstrated that the cell-free spent culture medium of *L. discophora* SS-1 is able to catalyze the oxidation of ferrocyanide to ferricyanide (6). The kinetics of this process, its temperature sensitivity, and its inhibition by enzyme poisons strongly suggested that iron oxidation in the spent medium is enzymatically catalyzed. We suggested that precipitation of iron oxides by *L. discophora* is mediated by one or more specific proteins. The iron-oxidizing enzyme(s) was not identified, but the experiments indicated that probably two different factors were involved in manganese and iron oxidation (6).

In this study, we identified at least one iron-oxidizing factor in the spent culture medium and in the cell lysate of *L. discophora* SS-1 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We isolated a strain of *L. discophora* SS-1 which gave rise to white colonies on manganese(II)-containing plates. Iron oxidation in this strain was also studied. To investigate whether iron oxidation is a common feature of manganese-oxidizing bacteria, a rapid test to screen for both activities was developed.

MATERIALS AND METHODS

Organisms and their cultivation. *L. discophora* SS-1 was kindly provided by W. C. Ghiorse (Cornell University, Ithaca, N.Y.). *Sphaerotilus natans* strains 565 and 566 were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Federal Republic of Germany. An uncharacterized manganese-oxidizing *Pseudomonas* sp. was provided by K. H. Nealson (Center for Great Lakes Studies, University of Wisconsin, Milwaukee, Wis.). Here it will be called strain GB-1. A manganese-oxidizing *Pseudomonas* sp., called strain GB-2, was isolated from a small creek in Utrecht, The Netherlands. All bacterial species were cultivated as described previously for *L. discophora* SS-1 (3, 4).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted by the method of Laemmli (12). Spent media of the *Leptothrix* sp. components were prepared for electrophoresis after coprecipitation of their components with manganese oxide as described previously (6). Cell lysates were prepared after collecting the cells from 100-ml early-stationary-phase cultures by centrifugation. The cell pellets were resuspended in 5 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 5 μl of a saturated solution of phenylmethylsulfonyl fluoride in isobutanol was added. The suspensions were sonicated 10 times for 10 s each under cooling in ice and then centrifuged at 15,000 × *g* for 10 min. Samples (2.5 ml) of the supernatants

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were desalted on a Sephadex G-25 column (PD-10; Pharmacia). The eluates were lyophilized and dissolved in 100 μl of Laemmli sample buffer (12) for electrophoresis. After electrophoresis, gels were stained for proteins with Coomassie brilliant blue, for manganese-oxidizing activity with an $MnCl_2$ solution as described before (4), or for iron-oxidizing activity as follows. Gels were rinsed with 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.0, for 45 min. The rinsing buffer was refreshed at least five times. Gels were then incubated in a freshly prepared solution of 2 mM $(NH_4)_2Fe(SO_4)_2$ in 10 mM MES, pH 6.0. Staining was stopped by rinsing the gels in demineralized water.

Protein determination. Protein contents were determined by the assay of Bradford (5). Bovine serum albumin was used as a standard.

Determination of manganese- and iron-oxidizing activity. A rapid semiquantitative assay for the determination of manganese- and iron-oxidizing activity in spent culture media and in cell lysates was developed. Spent media were used directly. Cell lysates were prepared as described above. Samples of known protein content were spotted in decreasing amounts on a nitrocellulose filter (Schleicher & Schuell; 0.45-μm pore size) in a Bio-dot Microfiltration Apparatus (Bio-Rad). The filter was removed from the apparatus and rinsed briefly in demineralized water. It was then incubated in a solution of 100 μM $MnCl_2$ in 10 mM HEPES, pH 7.5, or in a solution of 2 mM $(NH_4)_2Fe(SO_4)_2$ in 10 mM MES, pH 6.0. Manganese- and iron-oxidizing activity can be detected by the development of brown or yellow spots, respectively. No significant differences were found in the protein contents and metal-oxidizing activities of cell lysates before and after elution from the G-25 Sephadex column (see above). Therefore, this step was omitted in the preparation of the cell lysates for the dot-blot assay.

In some cases, the manganese-oxidizing activity was quantified with the Leucoberbelin blue assay (11).

RESULTS

Identification of an iron-oxidizing factor in *L. discophora* SS-1. The spent culture medium of *L. discophora* SS-1 contains manganese- as well as iron-oxidizing activity (6). It was shown before that a manganese-oxidizing factor can be identified after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 4). This factor is one of the components that coprecipitate with the manganese oxide formed from manganese chloride by active spent medium (4). A factor which promotes the oxidation of ferrous ions also coprecipitated with the manganese oxide. After gel electrophoresis of the solubilized coprecipitate, the iron-oxidizing factor could be visualized by incubating the gel in a solution of ammonium iron(II) sulfate at pH 6.0. This staining procedure resulted in a sharp band of precipitated iron oxide (Fig. 1, lane c). The iron-stained gel was then stained with Coomassie brillant blue (Fig. 1, lane d) and compared with a gel run with the same sample and directly stained for protein (Fig. 1, lane b). The iron-oxidizing factor appeared to be identical to a protein with an apparent molecular weight of approximately 150,000 (150K protein) (Fig. 1, lane b; compare lane a). Manganese oxidation at the position of the iron-oxidizing factor was hardly visible (Fig. 1, lane e), and vice versa, iron oxidation was hardly visible at the position of the manganese-oxidizing factor (Fig. 1, cf. lanes c and e). The absence of iron oxidation at the latter position was not due to inactivation of the manganese-oxidizing factor by the incubation at pH 6.0. At this pH, manganese was oxidized in the gel, albeit at a lower rate than at pH 7.5. Apparently, the spent medium contained an iron-oxidizing factor apart from the manganese-oxidizing factor identified before (3, 4).

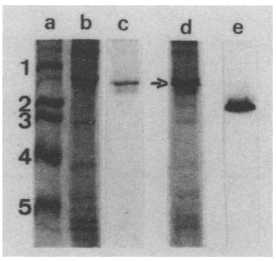

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of concentrated spent culture medium of *L. discophora* SS-1. Lanes: a, molecular weight markers (1, 205,000; 2, 116,000; 3, 97,000; 4, 66,000; 5, 45,000); b, spent medium, Coomassie brillant blue stained; c, spent medium, stained for iron-oxidizing activity; d, spent medium, stained for iron-oxidizing activity and then with Coomassie brillant blue (the arrow indicates the iron-stained band); e, spent medium, stained for manganese-oxidizing activity. Total protein applied in lanes b through e, 10 μg.

The oxidation of iron by molecular oxygen from the air proceeds at a higher rate than the oxidation of manganese under the conditions used. In principle, it is possible that the precipitation of iron oxide at the position of the 150K protein was the result of preferential binding of iron oxide formed by oxidation by air instead of by catalysis of the oxidation process. When the gel was incubated in a solution of ammonium iron(II) sulfate at a pH of 7.5, the ferrous ions were oxidized at a high rate by molecular oxygen, resulting in precipitation of iron oxide throughout the gel (result not shown). No preferential binding of the oxide at any position in the gel was observed, indicating that the iron oxide formed by the 150K protein resulted from catalysis of oxidation.

Iron and manganese oxidation by a spontaneous mutant of *L. discophora* SS-1. During subcultivation of *L. discophora* SS-1 on manganese(II)-containing plates, a white colony was observed among the colonies covered with manganese oxide. Apparently this colony did not oxidize manganese outside its cells. This raised the question of whether iron oxidation was also affected in these cells. The white cells were isolated and subcultivated as mutant strain 001 for investigation in greater detail. First, its interaction with manganese was characterized to some extent. For the sake of convenience, the original *L. discophora* strain will be called the wild type, in spite of the fact that it lost the sheath-forming ability by which it is characterized in nature.

Cultures of the *L. discophora* wild type and of the mutant strain 001 were grown simultaneously in liquid medium under the same conditions. Over 3 subsequent days, the OD_{660} was measured. The strains had similar growth rates. Each day, the manganese-oxidizing activity of the following samples was determined with the Leucoberbelin blue assay: the spent culture media after removal of the cells; the collected cells resuspended in 10 mM HEPES (pH 7.5) at the same cell density as the original cultures; and cells resuspended as above and lysed by ultrasonication, after which insoluble particles were removed by centrifugation (15,000 × *g*). In Table 1, the manganese-oxidizing activities of these samples of both the wild-type strain and mutant strain 001 are summarized. The data indicate that in the wild-type strain, the manganese-oxidizing factor was synthesized intracellularly and then transported through the cell membrane

TABLE 1. Manganese oxidation by *L. discophora* wild type and the mutant strain 001 in different growth phases

OD_{660}	Mn(II)-oxidizing activity (nmol/ml/min)							
	Wild type				Strain 001			
	Lysate	Cells	Medium	Total	Lysate	Cells	Medium	Total
0.40	1.8	1.5	1.1	4.4	4.7	0	0	4.7
0.55	3.3	5.0	0.8	9.1	5.0	0	0	5.0
0.66[a]								
Expt 1	0.7	2.0	6.7	9.4	6.7	0.2	0	6.9
Expt 2	0.5	0.7	2.9	4.1	4.6	0	0	4.6
Expt 3	0.4	1.0	4.7	6.1	5.0	0.2	0	5.2

[a] Early stationary phase.

and finally almost completely excreted into the culture medium. In mutant strain 001, the manganese-oxidizing factor was also synthesized intracellularly, and after lysis of the cells, (at least part of) the manganese-oxidizing activity could be determined. Contrary to the spent medium of the wild-type strain, the spent medium of mutant strain 001 did not show manganese-oxidizing activity. Apparently the excretion of the manganese-oxidizing factor through the cell membrane was inhibited, or the factor was excreted in an inactive form. The total manganese-oxidizing activity (the sum of the activities of lysate, cells, and medium) did not differ significantly between the wild type and mutant strain 001 (Table 1). The manganese-oxidizing activity associated with particulate material after lysis of the cells was ignored in these experiments. Preliminary studies of the distribution of the manganese-oxidizing activity in the lysates of the mutant and wild-type strains indicated that the activity associated with particulate material, as obtained by the procedure described above, can amount to 30% of the activity obtained in the soluble fraction. No difference in the distribution of the activities of particulate and soluble material in the lysates of the two strains has been observed so far.

The spent culture medium of mutant strain 001 had no iron-oxidizing activity. Only the cell lysate was capable of oxidizing iron. The cell lysate contained both an iron-oxidizing and a manganese-oxidizing factor, with electrophoretic migration rates similar to those of the factors from the spent medium of the wild-type strain (not shown). The absence of manganese- and iron-oxidizing activity in the spent medium of the white mutant strain 001 might be the result of inhibition of protein excretion in this strain. The protein contents of the spent culture media of early-stationary-phase cultures of mutant strain 001 and the wild-type strain were determined in two independent experiments. They amounted to a mean of 2.7 and 3.3 μg of bacterial protein per ml of spent medium, respectively. Apparently, mutant strain 001 was capable of protein secretion. This was confirmed by gel electrophoresis of lyophilized spent medium of mutant strain 001. The Coomassie brilliant blue-stained electropherogram obtained resembled that of the spent medium of the wild type (results not shown), but metal-oxidizing factors were not detected. The possible absence of protein bands as a result of the absence of both metal-oxidizing activities could not be clearly demonstrated. Owing to the low protein content of the spent medium, concentration of the components prior to electrophoresis was necessary. Because the spent medium of mutant strain 001 lacked metal-oxidizing activity, concentration of excreted products by coprecipitation with metal oxides was not possible. By freeze-drying, the excess nutrient components of the culture medium were also concentrated. They were not easily separated from the excreted cellular products by column chromatography or dialysis and interfered with the electrophoretic mobility of the latter.

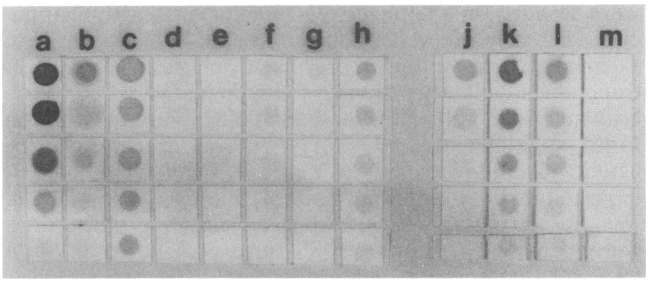

FIG. 2. Dot-blot assay for manganese- and iron-oxidizing activity by different bacterial species. Samples of spent media (a and d) and cell lysates (b, c, e through m) were diluted by a factor of 1, 2, 4, 8, and 16, and these samples were spotted on a nitrocellulose filter. The filters were rinsed and stained for manganese (a through h) or iron (j through m) oxidation activity. Bacterial species: a, b, and j, *L. discophora* wild type; c, d, and k, *L. discophora* mutant strain 001; e and l, *S. natans* 565; f, *Pseudomonas* sp. strain GB-2; g, h, and m, *Pseudomonas* sp. strain GB-1. Protein concentration: undiluted spent media, 2 μg/ml; undiluted cell lysates, 1 mg/ml.

Iron and manganese oxidation by other bacterial species. *L. discophora* is able to oxidize both iron and manganese. Microbial oxidization of manganese has been studied more extensively than microbial oxidation of iron under conditions of more or less neutral pH. In order to investigate whether bacteria that are able to oxidize manganese will also oxidize iron, a dot-blot assay was developed for rapid screening of different bacterial species. When the protein contents of comparable samples (e.g., spent media or cell lysates) are known and dilution series of the samples are tested for manganese- or iron-oxidizing activity, the lowest protein concentration, i.e., the highest dilution factor, at which oxide formation is observed can be considered a crude measure of the activity. In Fig. 2, an example of such an assay is presented. Rows a to h represent manganese oxidation, and rows j to m represent iron oxidation. The activity of spent culture media from the wild-type strain and mutant strain 001 of *L. discophora* SS-1 is shown in a and d, respectively. All other samples represent dilution series of cell lysates (starting concentration, 1 mg of protein per ml). As expected, lysates of early-stationary-phase cells of *L. discophora* wild type (b and j) contained significantly less metal-oxidizing activity than the cell lysate of mutant strain 001 (c and k; see also Table 1). *S. natans* is a sheath-forming bacterial species related to *L. discophora* (14). *S. natans* 565 did not show manganese-oxidizing activity in the cell lysates (e), not even when 10× concentrated samples were tested, but the cell lysates were capable of iron oxidation (1). The *Pseudomonas* sp. strains GB-2 (f) and GB-1 (g) showed only slight manganese-oxidizing activity at the concentrations used, but the activity was enhanced more or less proportionally when a 10× concentrated sample was tested (h, *Pseudomonas* sp. strain GB-1). No iron oxidation was detected in this concentrated sample (m).

In Table 2, the results of the dot-blot assay obtained with different bacterial species are summarized. In all cases, early-stationary-phase cell cultures were used. Of the species tested, *L. discophora* was the only one able to oxidize both iron and manganese. The spent media of most of the strains contained proteins, as determined by the Bradford

TABLE 2. Manganese and iron oxidation in spent culture media and cell lysates of some bacterial species

Species[a]	Protein concn (μg/ml) in spent medium	Oxidizing activity[b] (dilution factor)			
		Spent medium		Cell lysate[c]	
		Mn	Fe	Mn	Fe
L. discophora wild type	2	16	8	4	2
L. discophora 001	2	—	—	16	16
Pseudomonas sp. strain GB-1	3	—	—	1	—
Pseudomonas sp. strain GB-2	3	—	—	2	—
S. natans 565	2	—	—	—	8
S. natans 566	0.7	—	—	—	—
E. coli Y1090r$^-$[d]	ND[e]	—	—	—	—

[a] Early-stationary-phase cultures were used.
[b] Metal-oxidizing activities are represented by the highest factor by which samples could be diluted and still show visible oxide formation in the dot-blot asay. —, no activity at any dilution.
[c] Prior to the assay, cell lysates were diluted to a protein concentration of 1 mg/ml.
[d] *E. coli* Y1090r$^-$ was obtained from Promega Corporation.
[e] ND, not determined.

assay, but only the wild-type strain of *L. discophora* excreted iron- and manganese-oxidizing activities into the culture medium. In the other species, the metal-oxidizing activities, if present, were associated with the cells. The *Pseudomonas* species oxidized only manganese, and *S. natans* 565 oxidized only iron. The latter strain formed long strings of sheaths, which could be clearly recognized under the light microscope, especially after incubation of the cells with Alcian blue (7% [wt/vol] in 2% [vol/vol] acetic acid). Alcian blue clearly stained the sheaths. Strain 566 produced far fewer sheath-enveloped cell strings, and single cells devoid of sheaths, as observed after Alcian blue staining, formed the majority of the cell population in these cultures. Iron oxidation by cell lysates of strain 566 was not observed with the dot-blot assay, suggesting a relationship between iron oxidation and sheath formation in *S. natans*. A strain of *Escherichia coli* served as a control in the dot-blot experiments. No metal-oxidizing activity was detected.

As stated above, part of the metal-oxidizing activity of *L. discophora* was contained in the particulate material after ultrasonication of the cells. We checked the possibility that the absence of metal-oxidizing activity in some of the samples as observed in the dot-blot assay was due to the lack of solubilization of metal-oxidizing factors. Therefore, the corresponding particulate fractions were also assayed (maximum amount of protein tested per sample, about 100 μg). In no case was metal oxidation detected.

DISCUSSION

In a previous study, we demonstrated oxidation of iron by the spent culture medium of *L. discophora* SS-1 (6). The experiments indicated that enzymatic activity was involved. In the present study, an iron-oxidizing factor was identified after electrophoretic fractionation of the spent culture medium. The activity coincided with a protein with an apparent molecular weight of approximately 150,000. When the gel was incubated in a solution of the redox indicator *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), blue staining as a result of the formation of Wurster blue was observed at the same position (6). The iron-oxidizing factor described here is identical to the TMPD-oxidizing factor described in the previous work. The fact that the TMPD-oxidizing factor was not identified as an iron-oxidizing factor before was probably the result of the use of the ferrocyanide complex as a substrate for the reaction. The resulting ferricyanide is soluble, and apparently it diffused away and did not reside at the reaction site long enough to allow localization of the reaction in the polyacrylamide gel. The use of ammonium ferrosulfate as a substrate for the staining reaction as presently described results in an insoluble oxide. The pH at which this staining is performed is important; under neutral or alkaline conditions, the ferro ions are rapidly oxidized by air, and biological oxidation cannot be distinguished from chemical oxidation. Biological iron oxidation was also not observed at pH 5 (or lower), possibly due to inactivation of the iron-oxidizing factor.

The fact that two different macromolecules, capable of iron and manganese oxidation, respectively, could be distinguished by sodium dodecyl sulfate-polyacrylamide gel electrophoresis supports the previous conclusion that different factors are involved in the oxidation of these metals (6). But the two processes also share several properties. Both activities could still be detected after subjection of the metal-oxidizing factors to denaturing conditions. Both were accumulated in the cells of mutant strain 001. In this strain, excretion of the metal-oxidizing factors appeared to be inhibited, whereas the excretion of other proteins seemed not to be impaired. This suggests a close link between excretion of the iron- and manganese-oxidizing factors. Although further research is needed to reveal the nature of the mutation(s) in mutant strain 001, it is tempting to speculate that the iron- and manganese-oxidizing factors are excreted by similar mechanisms. It remains uncertain whether the two metal-oxidizing factors have a similar or a coupled function. In principle, energy can be obtained from the oxidation of both iron and manganese. So far, chemolithotrophic growth on iron has been demonstrated for acidophilic iron oxidizers (9) and suggested for members of the neutrophilic genus *Gallionella* (8). Growth on manganese as a single source of energy was demonstrated for a manganese-oxidizing *Pseudomonas* species (10).

Manganese and iron oxidation abilities are widely spread among microbial genera. The dot-blot assay described here is a rapid semiquantitative test for screening the metal-oxidizing ability of different microorganisms. In this assay, the samples are concentrated on a small area of a nitrocellulose filter. When the ratio between the concentrations of the oxidizing factors and other macromolecules, especially those binding to nitrocellulose, is low, part of the oxidizing activity may be masked by the other components. Therefore, only equivalent samples (i.e., spent media or cell lysates) can be compared directly for their oxidizing activity. The quantitative comparison of the total manganese-oxidizing activities of *L. discophora* SS-1 wild type and mutant strain 001 (Table 1) included the summation of the activities measured in the spent medium and in the cell lysate of one strain by the Leucoberbelin blue assay. In this assay, direct comparison of these activities seems justified because they are determined in solutions which are relatively diluted.

The ability to oxidize iron, manganese, or both may be a specific feature of a bacterial species. The question arises whether common principles underlie the metal-oxidizing processes in different organisms. *L. discophora* is the only species in which both a manganese- and an iron-oxidizing factor have been identified. So far, we have not been

successful in correlating the metal-oxidizing activity in any of the other species with one or more of their (macro)molecules. Apparently the techniques applied to date do not readily reveal common principles and components involved in the oxidation of iron and manganese in different organisms. The identification of the two metal-oxidizing factors from *L. discophora* may open up the way for a new approach. The isolation of the genes coding for these factors will allow the construction of DNA probes which can be used to trace homologous sequences in the DNA of other metal oxidizers.

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