Bacteriology of Manganese Nodules

V. Effect of Hydrostatic Pressure on Bacterial Oxidation of Mn\textsuperscript{II} and Reduction of MnO\textsubscript{2}

H. L. EHRLICH

Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181

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It was experimentally demonstrated that two strains of *Arthrobacter* 37, one growing at 25 C and the other at 5 C, could catalyze Mn\textsuperscript{II} oxidation at hydrostatic pressures well in excess of the pressure encountered by the parent culture in its original habitat in the ocean (80 atm). The strain grown at 5 C showed an increase in temperature optimum for manganese oxidation with increase in pressure. It was likewise experimentally shown that induced *Bacillus* 29 without added ferricyanide and uninduced *Bacillus* 29 with added ferricyanide could catalyze MnO\textsubscript{2} reduction at hydrostatic pressures in excess of the pressure encountered by this organism in its original habitat (187 atm). The uninduced *Bacillus* 29, in the presence of ferricyanide, was active over a wider range of pressures (1 to 1,000 atm) than the induced *Bacillus* 29 in the absence of ferricyanide (1 to 457 atm). At corresponding pressures, the uninduced culture was also considerably more active than the induced culture. Special techniques were developed for measuring Mn\textsuperscript{II}-oxidizing and MnO\textsubscript{2}-reducing activity under pressure.

Ferromanganese nodules in the marine environment occur mostly at depths below 90 meters (11). Evidence has been developed which suggests that microbes participate in the genesis and degradation of these nodules (2-4, 14, 15). On the basis of this work, the simplest mechanism of nodule genesis can be explained as an initial adsorption of Mn\textsuperscript{II} to a nucleus such as a micronodule, a pumice grain, a phosphorite grain, an ear bone of a whale, a shark’s tooth, or some other object at the sediment interface, followed by oxidation of the adsorbed Mn\textsuperscript{II} to manganic oxide (e.g., H\textsubscript{2}MnO\textsubscript{3}). The manganic oxide then reacts with additional Mn\textsuperscript{II} to form MnMnO\textsubscript{2},

\[ \text{Mn}^{II} + \text{H}_2\text{MnO}_3 \rightarrow \text{MnMnO}_3 + 2\text{H}^+ \]  \hspace{1cm} (1)

after which the MnMnO\textsubscript{2} is oxidized to manganic oxide by oxygen,

\[ \text{MnMnO}_3 + \frac{1}{2}\text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2\text{MnO}_3 \]  \hspace{1cm} (2)

I have shown that some bacteria, like *Arthrobacter* 37, associated with ferromanganese nodules, can catalyze reaction 2 enzymatically (4). This bacterial activity gains in importance as the nodule mass increases and the oxidation step becomes the rate-limiting step in nodule growth. I believe that iron, which is also a major constituent of marine ferromanganese nodules (1), is incorporated in ferric form by adsorption to or ion exchange with H\textsubscript{2}MnO\textsubscript{3}. Ferric, but not ferrous iron, stimulates the Mn\textsuperscript{II}-oxidizing activity of *Arthrobacter* 37 for some unknown reason when manganic oxide but not ferrimanganic oxide adsorbent is used (H. L. Ehrlich, unpublished data). A similar adsorption or ion-exchange mechanism is visualized for the incorporation of some trace metal (e.g., copper, nickel, cobalt, and zinc). Goldberg (5) proposed in 1954 that the hydroxides of manganese and iron in ferromanganese nodules could scavenge some of the metallic elements in seawater. Ehrlich, on the other hand, has presented evidence that rare earths are incorporated partly by surface transfer from detritus and partly by occlusion of detritus (A. M. Ehrlich, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, 1968).

Bacteria may thus regulate nodule growth by controlling the rate of formation of new adsorption or ion-exchange sites in their oxidation of MnMnO\textsubscript{2} to H\textsubscript{2}MnO\textsubscript{3}. Other workers have proposed different roles for microbes in nodule genesis, but their evidence is limited (6, 7, 9). Formation of ferromanganese nodules without microbial intervention has also been proposed by a number of investigators. Such theories have been summarized by Mero (12).

The work of Trimble and Ehrlich shows that nodule degradation by microbes, when it occurs, is frequently enzymatic. In this process, the orga-
nisms catalyze the reduction of the manganic oxide in the nodule by a suitable electron donor, such as glucose (14, 15). Such reduction results in extensive solubilization of the manganese as Mn\(^{2+}\), but not of the iron (H. L. Ehrlich, Bacteriol. Proc., p. 42, 1964). The fate of the trace elements will be the subject of a separate communication (Ehrlich, Yang, and Mainwaring, in preparation).

The MnO\(_2\) reductase system with which Bacillus 29 reduces the manganic oxide component in ferromanganese nodules has been studied by Trimble and Ehrlich (15). A portion of this enzyme system is inducible by Mn\(^{2+}\). Putatively similar inducible MnO\(_2\) reductases have been found in some other bacterial isolates from ferromanganese nodules (H. L. Ehrlich, unpublished data).

Up to now, the experimental evidence which supports a bacterial role in the genesis and degradation of ferromanganese nodules has been obtained at atmospheric pressures. Since, however, in the marine environment these reactions must proceed at hydrostatic pressures corresponding to the depths at which the nodules are found, it became important to show that the microbial reactions contributing to nodule genesis and degradation can proceed at such pressures. This paper presents evidence that this is possible.

**MATERIALS AND METHODS**

**Cultures.** A strain of *Arthrobacter* 37 grown at 25 C (NCIB 10328, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland) and one grown at 5 C were used in the manganese-oxidation studies (3). Bacillus 29 (3, 14, 15), grown at 25 C, was used in the MnO\(_2\)-reduction studies. The cultures were maintained on test tube slants of seawater-nutrient agar (4).

Mass cultures of each strain were prepared on Roux slants of seawater-nutrient agar (4). The *Arthrobacter* 37 strain grown at 25 C was cultured on two Roux slants for 16 to 17 hr, and the strain grown at 5 C was cultured on three Roux slants for 1 week. Bacillus 29 was grown on two Roux slants for 24 hr at 25 C. The cells on the slants were harvested in 10 ml of seawater and washed three times with 10 ml of seawater by centrifugation at 14,500 \(\times g\). The final suspension of each culture was adjusted to contain in the order of 10\(^8\) cells per ml, as determined by turbidimetry. The harvesting and washing of the strain grown at 5 C was with seawater prechilled to 5 C.

Mass-induced cultures of Bacillus 29 were prepared according to the method of Trimble and Ehrlich (15).

**Impregnation of absorption pads with manganic oxide.** Enzymatic oxidation of Mn\(^{11}\) by bacteria depends on intimate contact between appropriate bacterial cells and manganic oxide with adsorbed Mn\(^{2+}\). Similarly, enzymatic manganic oxide reduction depends on intimate contact between appropriate bacterial cells and the oxide. Since the reaction vessels for the pressure experiments were upright test tubes, solid manganous substrate placed on the bottom of such tubes would expose too limited a surface for activity to be detected after 4 hr of incubation. To increase the surface exposed by the solid manganous substrates, absorption pads (Millipore Corp., Waltham, Mass.) were impregnated with them and draped around the wall of the tube, by use of the following procedure based on a suggestion by J. V. Landau (Rensselaer Polytechnic Institute).

For Mn\(^{11}\)-oxidation experiments, 0.1 g of Mn-Fe oxide (–260 mesh) synthesized according to the method of Ehrlich (3), or, for MnO\(_2\) reduction experiments, 0.1 g of finely powdered, reagent-grade MnO\(_2\) (J. T. Baker Chemical Co., Phillipsburg, N.J.), was placed on a preweighed absorption pad (47-mm diameter; Millipore Corp.) in a Millipore filter assembly. A 50-ml amount of distilled water was then added to the filter. The manganous substrate was suspended in it by swirling and then allowed to deposit evenly over the surface of the absorption pad while the water was pulled through the pad by suction. Residual oxide on the walls of the funnel was rinsed onto the pad with an additional 10 ml of distilled water. The pad, with the manganous-substrate carrying side facing up, was then transferred from the filter to the bottom of a 250-ml beaker and covered with 10 ml of distilled water. After 90 min, the pad was rinsed under a stream of distilled water to free it from loose manganous substrate and then was dried in its beaker in an oven at 80 to 90 C for 30 min or until just dry. It was important not to dry the pad excessively because of chemical reaction between the pad and the manganic oxide. Pads impregnated with Mn-Fe oxides usually gained 2.4 ± 0.2 mg in weight, and pads impregnated with MnO\(_2\) usually gained 9.9 ± 0.2 mg in weight. All pads needed for a given experiment were always prepared together. Prepared pads could be stored up to 3 days before being used in pressure experiments. Impregnated pads stored for longer times did not work, for reasons that are not understood.

**Deposition of bacteria on impregnated pads.** In typical pressure experiments, appropriate bacteria were deposited on four of eight impregnated pads. To deposit the cells, a pad, with the manganous-substrate impregnated side facing up, was mounted in a Millipore filter assembly. A 5-ml amount of a bacterial seawater suspension containing a known number of cells (in the order of 10\(^9\)) was then added to the filter and pulled through the pad by suction. It was found experimentally that, of about 10\(^9\) cells of *Arthrobacter* 37, approximately 25% were retained by a pad, and, of about 10\(^9\) cells of Bacillus 29, approximately 58% were retained by a pad.

The pads on which bacteria were not deposited were also moistened by pulling 5 ml of seawater through them in a Millipore filter assembly.

**Setting up of the reaction tubes.** While still moist, each pad was draped around the inside wall of a test tube (65 by 18 mm) with the manganous-substrate impregnated side facing inward. Each tube was then filled with 10 ml of an appropriate reaction solution.
For the oxidation reaction, the solution consisted of seawater containing 0.001 M MnSO₄·H₂O and 1.14 × 10⁻² M NaHCO₃ (pH 7.0). For the MnO₂ reduction reaction, the solution consisted of 12.8-fold diluted seawater containing 7.8 × 10⁻⁴ M glucose, 3.4 × 10⁻² M NaHCO₃ (pH 7.0), and, when required, 1.4 × 10⁻⁴ M K₃Fe(CN)₆. Each tube was then stoppered with a n. 0 rubber stopper, with care taken to avoid entrapment of any air bubble between the stopper and the liquid.

In the case of the Arthrobacter 37 strain grown at 5 C, the reaction solution was chilled to 10 C before introduction to the tubes.

**Pressurization of reaction tubes.** Reaction tubes were pressurized in the vessel shown in Fig. 1. The design of the vessel is based on a general one presented by ZoBell and Oppenheimer (17). The inside lumen of the vessel had a diameter of 6.5 cm and a height, when closed, of 14.8 cm. The wall of the vessel had a thickness of 2.3 cm. A thermistor probe was inserted through the side of the vessel, 9 cm above the inside bottom (A, Fig. 1).

For measurements of activity at a given hydrostatic pressure, eight reaction tubes were divided into two sets. Each set consisted of two tubes containing manganese-substrate impregnated pads with cells and two tubes containing manganese-substrate impregnated pads without cells. One of these two sets was placed on a brass carrier (B, Fig. 1) and placed inside the pressure vessel filled with paraffin oil. For experiments with the strain of Arthrobacter 37 grown at 5 C, the pressure cell was prechilled to 5 C before introduction of the reaction tubes. After placing the carrier with the reaction tubes into the pressure vessel, it was closed and pressurized with a hydraulic pump; paraffin oil was used as the pressure-transmitting fluid. The assembly and pressurization, whether for experiments around 25 C or around 10 C, were always carried out at room temperature. During this time, the internal temperature of the chilled pressure cell approached 10 C as measured with the thermistor probe. For experiments around 10 C, the pressurized cell was immediately wrapped with Calorex tubing (Cole Parmer Instrument Co., Chicago, Ill.) through which water coolant at a suitable temperature (9 to 13 C) was circulated to maintain the pressure cell at a desired temperature. Temperature equilibration in the pressure vessel between 10 and 14 C was achieved within 5 to 15 min. Temperature equilibration in the pressure vessel at room temperature was achieved in about 3 min. The second set of four tubes prepared for each pressure experiment was incubated at atmospheric pressure and at a desired temperature. All tubes were incubated for 4 hr. The pressure in the pressure vessel was then checked and released. The liquid content of all reaction tubes was then analyzed for Mn²⁺ concentration.

**Chemical analyses.** In oxidation experiments, two 1-ml samples were analyzed directly for Mn²⁺ by the persulfate method for short-term manganese oxidation (3). In reduction experiments, 1.5 ml of reaction solution was first removed from each tube, and the remaining solution was then acidified by adding 0.05 ml of 10 N H₂SO₄ and mixing with a pipette. Thirty min later, two 1-ml portions were analyzed for Mn²⁺ by the persulfate method for short-term MnO₂ reduction (3).

**RESULTS**

**Manganese oxidation around 25 C.** The extent of manganese oxidation catalyzed by the strain of Arthrobacter 37 grown at 25 C, after 4 hr, was determined at room temperature between 1 and 567 atm. As in previous work (4), the oxidation was measured in terms of the difference in residual Mn²⁺ concentrations (∆Mn) between reaction tubes with and without bacterial cells. Typical results are shown in Table 1. It can be seen that bacterial manganese oxidation ceased between 467 and 567 atm. The activity at 467 atm and below was not significantly affected by hydrostatic pressure.

**Manganese oxidation around 10 C.** The extent of manganese oxidation catalyzed by the strain of Arthrobacter 37 grown at 5 C, after 4 hr, was

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**Table 1.** Hydrostatic pressure effects on Mn²⁺ oxidation by Arthrobacter 37 grown at 25 C.

| Pressure (psi) | Temp (C) | Cells (x 10⁸) | ∆Mn (atm) (control) |
|---------------|----------|---------------|---------------------|
| 5,000         | 23.0     | 10            | 1                   |
| 7,000         | 23-24    | 7.5 x 10⁸     | 1                   |
| 8,500         | 24.5     | 9 x 10⁸       | 1                   |

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**FIG. 1.** Hydrostatic pressure-cell assembly. (A) Thermistor probe. (B) Brass carrier. (C) Reaction tubes. (D) Impregnated absorption pad.
determined at atmospheric pressure and at various elevated hydrostatic pressures at temperatures between 10 and 14 C. As before, the oxidation was measured in terms of the difference in residual Mn^{II} concentration (ΔMn) between reaction tubes with and without cells. As shown in Table 2, bacterial manganese oxidation at 1 atm disappeared between 10 and 13 C (controls). Other experiments have shown that for Mn^{II} oxidation by this culture the temperature optimum at 1 atm lies around 4 C. Activity falls off only slightly as the temperature approaches 10 C. Table 2 also shows that manganese oxidation occurred between 10 and 12 C at hydrostatic pressures up to and including 200 atm. However, at pressures of 266 atm and above, manganese oxidation occurred at 13 and 14 C but not at 10 C. These results indicate that the temperature optimum of the manganese-oxidizing system in the bacteria rose with increased hydrostatic pressure.

**MnO_2 reduction by uninduced Bacillus 29.** The reduction by uninduced Bacillus 29 was determined in the presence of ferricyanide between 1 and 1,000 atm at room temperature. The MnO_2-

| Pressure | Temp (°C) | Cells | ΔMn (nmoles/ml) |
|----------|-----------|-------|-----------------|
| | | | At pressure | Control (1 atm) |
| psi | atm | | |
| 2,000 | 133 | 10 | 9 × 10^8 | 30 | 35^a |
| 2,000 | 133 | 13 | 10 × 10^8 | 0 | 0 |
| 3,000 | 200 | 11 | 9 × 10^8 | 20 | 30 |
| 3,000 | 200 | 12 | 9 × 10^8 | 25 | 25^a |
| 4,000 | 200 | 13 | 10 × 10^8 | 0 | 0 |
| 4,000 | 266 | 9-10 | 9 × 10^8 | 0 | 20^a |
| 4,000 | 266 | 13 | 9 × 10^8 | 30 | 0 |
| 5,000 | 333 | 10 | 10 × 10^8 | 0 | 20^a |
| 5,000 | 333 | 13-14 | 10 × 10^8 | 65 | 40^a |
| 10,000 | 667 | 14 | 10 × 10^8 | 55 | 0 |
| 15,000 | 1,000 | 14 | 10 × 10^8 | 20 | 30^a |

^a These controls were incubated at 10 C.

**DISCUSSION**

The ferromanganese nodule from which the *Arthrobacter* 37 parent strain was isolated came from a depth of about 730 meters in the Atlantic Ocean, where the corresponding hydrostatic pressure is about 80 atm. The ferromanganese nodule from which *Bacillus* 29 was isolated came from a depth of about 1,700 meters, where the corresponding hydrostatic pressure is about 187 atm. The results from the present study show that *Arthrobacter* 37 and *Bacillus* 29 can carry out manganese oxidation and reduction, respectively, at the pressures prevailing in their original habitats, and, indeed, can perform these activities at even higher pressures.

Of the *Arthrobacter* strains and the *Bacillus* strain, only the *Arthrobacter* strain grown at 5 C was active in a temperature range corresponding to that which might have prevailed in its original habitat. The strain of *Arthrobacter* 37 grown at 25 C has a temperature optimum for Mn^{II} oxidation of 17.5 C at atmospheric pressure (18). *Bacillus* 29 has a temperature optimum for MnO_2 reduction of 18 C at atmospheric pressure (15). Both of the latter organisms are inactive at 10 C under atmospheric pressure. The temperature ranges for their respective activities are thus well above those most likely prevailing in their original habitats. Since

| Pressure | Temp (°C) | Cells | Mn released (nmoles/ml) |
|----------|-----------|-------|------------------------|
| | | | At pressure | Control^a |
| psi | atm | | |
| 5,000 | 333 | 24.5-26 | 2.7 × 10^8 | 510 |
| 7,000 | 467 | 24.8 | 2.8 × 10^8 | 295 |
| 10,000 | 667 | 25-26 | 2.8 × 10^8 | 115 |
| 15,000 | 1,000 | 24.5-25.5 | 2.9 × 10^8 | 54 |

^a Run at atmospheric pressure.
these cultures were originally isolated from nodules at 25°C as a matter of convenience and have been subcultured at this temperature for over 9 years, they probably have changed their temperature characteristics during this time. It will be desirable to repeat the MnO$_2$ reduction experiments with cultures whose temperature optimum is in the vicinity of temperatures prevailing in their natural marine habitat.

The change in temperature optimum with increase in hydrostatic pressure which was observed in the case of Mn$^{II}$ oxidation by the strain of Arthrobacter 37 grown at 5°C is not an unusual phenomenon. Similar observations have been made with other enzyme-catalyzed activities (e.g., 8, 10, 13, 16). On the assumption that the manganese-oxidizing and -reducing enzymes are located at the cell surface (plasma membrane), these phenomena can be explained on the basis that hydrostatic pressure tends to interfere with a necessary increase in molecular volume because of enzyme-substrate interaction and that this effect of pressure can be counteracted by an appropriate increase in temperature (8).

The range of pressures at room temperature at which induced Bacillus 29 was able to reduce MnO$_2$ was much narrower (1 to 467 atm) than for the uninduced culture in the presence of ferricyanide. The more intense MnO$_2$-reducing activity of uninduced Bacillus 29 in the presence of ferricyanide compared with induced Bacillus 29 in the absence of ferricyanide at corresponding pressures suggests that ferricyanide is a more efficient electron carrier than the natural, inducible electron-carrying component of Bacillus 29.

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**TABLE 4. Hydrostatic pressure effects on Mn$^{IV}$ reduction by induced Bacillus 29**

| Pressure (psi) | temp (°C) | Mn released (nmol/ml) |
|---------------|-----------|-----------------------|
| 3,000         | 22-23     | 14                    |
| 5,000         | 23.5      | 16                    |
| 6,000         | 24.5      | 9                     |
| 7,000         | 23.5      | 0                     |

* The number of cells in each experiment was 2.2×10$^8$.

* Run at atmospheric pressure.

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