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Cytochrome Involvement in Mn(II) Oxidation by Two Marine Bacteria

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Two marine, Mn(II)-oxidizing bacterial cultures, BIII 45 and BIII 82, were examined spectrophotometrically at ambient temperature for their cytochrome complements. Membrane preparations from an ethylenediaminetetracetate-lysozyme treatment of 48-h cultures of both strains contained type *b*, *c*, and *o* cytochromes. No evidence for a type *a* cytochrome was noted. "Periplasmic" fractions of both strains also contained small amounts of cytochrome, including cytochrome *o*, but "intracellular" fractions did not. Type *c* cytochrome in membrane preparations of culture BIII 45 was consistently reduced by Mn(II) when the membranes were suspended in the periplasmic fraction of the culture. In the case of culture BIII 82, type *c* cytochrome in membrane preparations was consistently reduced by Mn(II) when the membranes were suspended in either periplasmic or intracellular fractions of the strain. Although, based on previous inhibitor studies, type *b* cytochrome was also expected to be reduced by Mn(II), no spectrophotometric evidence for its reduction was found, probably because not enough of it was reduced under the steady-state conditions of the experiments.

Bacteria which have the ability to oxidize Mn(II) are common in marine sediments (9). Their oxidation of Mn(II) has been found to be enzymatic (2). All of the bacteria studied by Ehrlich were able to oxidize Mn(II) only after it had first been adsorbed to particulate manganic oxides. He, therefore, proposed the following reaction sequence to explain their action:

$$H_2MnO_3 + Mn(II) \xrightarrow{\text{nonenzymatic}} MnMnO_3 + 2H^+ \quad (1)$$

$$MnMnO_3 + 2H_2O + 1/2\ O_2 \xrightarrow[\text{peptone or } NaHCO_3]{\text{bacteria}} (H_2MnO_3)_2 \quad (2)$$

In an excess of Mn(II), the second of the two reactions is the slower, which means that the bacterial oxidation is the rate-limiting reaction in this sequence.

It has been postulated that marine Mn(II)-oxidizing bacteria can grow mixotropically, using the oxidation of manganese to supply part of their energy needs (4). Evidence that supports this hypothesis comes from studies of whole cells and cell extracts of a gram-negative, deep-sea, Pacific Ocean isolate, culture BIII 45 (5). When Mn(II) was added to suspensions of whole cells of this organism, it stimulated their uptake of radioleucine. This stimulation was phosphate dependent. Although these results do not directly prove that useful energy is derived from the oxidation of Mn(II), they do suggest it. More direct evidence was derived from experiments in which Mn(II) oxidation by cell extracts of culture BIII 45 was directly coupled to a net synthesis of adenosine 5'-triphosphate (5). Ratios of adenosine 5'-triphosphate produced to Mn(II) removed by strain BIII 45 ranged from 0.032 to 0.101.

The coupling of adenosine 5'-triphosphate synthesis to Mn(II) oxidation by culture BIII 45 has been thought to involve a typical electron transport chain since dicumarol, antimycin A, 2-*n*-nonyl-4-hydroxyquinoline-*n*-oxide, cyanide, and azide have all been found to inhibit Mn(II) oxidation to one extent or another (5). These findings have suggested the possible involvement of flavins, quinones, type *b* cytochromes, type *c* cytochromes, and a cytochrome oxidase in the oxidation of Mn(II), and they have suggested further that as many as 2 mol of adenosine 5'-triphosphate could be produced per mol of Mn(II) oxidized.

The present paper reports spectrophotometric evidence for the presence of cytochromes in two strains of marine Mn(II)-oxidizing bacteria in Ehrlich's collection and also spectrophotometric evidence for the involvement of cytochromes in the oxidation of Mn(II).

MATERIALS AND METHODS

Cultures. Bacterial cultures BIII 45 and BIII 82 were used in this study. They are both gram-negative,

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deep-sea, Pacific Ocean isolates which resemble *Oceanospirillum* (E. P. White, unpublished data). Whereas both strains are able to carry out the enzymatic oxidation of Mn(II), they differ from each other in various other physiological traits.

Stock cultures of each strain were maintained by monthly transfer on test tube slants of nutrient agar (Difco), made up in full-strength, filtered seawater. Slants inoculated with either culture were incubated and stored at 15°C. Working cultures were derived from stock cultures by transfer to fresh seawater-nutrient agar slants in test tubes and incubation for 24 h at 15°C. These 24-h cultures were used to inoculate five seawater-nutrient agar slants in Roux bottles. The inoculated Roux slants were incubated at 15°C for 48 h.

Starvation procedure. Cultures, grown up on Roux slants, as described above, were harvested and washed three times in 40.0 ml of sterile, full-strength seawater by centrifugation at 12,000 × *g* for 5 min at 4°C. The final pellet was resuspended in 10 to 20 ml of sterile, full-strength, filtered seawater and aseptically starved by forced aeration for 16 h at 15°C.

Preparation of cell fractions by EDTA-lysozyme treatment of whole cells. All reagents for this fractionation, except $NaHCO_3$ buffer, were prepared in 3% NaCl solution. The pH of the tris(hydroxymethyl)aminomethane-hydrochloride buffer (Tris buffer) (Sigma Chemical Co.), the $NaHCO_3$ buffer, and the disodium(ethylenedinitrilo)tetraacetate (EDTA) solution (Baker) was adjusted to 7.5. The Tris buffer and EDTA solution were chilled to 4°C, and the $NaHCO_3$ buffer was chilled to 15°C.

Cells were grown, harvested, and washed as described above. The final pellet of cells was resuspended in 10.0 ml of sterile seawater and centrifuged at 12,000 × *g* for 10 min at 4°C. The resulting pellet was resuspended in 15 ml of 3×10^{-2} M Tris buffer. The cell suspension was then allowed to equilibrate at 15°C for 30 min. A 5.0-ml volume of EDTA solution was now added to the cell suspension with mixing, followed about 15 s later by 5.0 ml of a solution of lysozyme (Sigma Chemical Co.) at a specified concentration. This mixture was incubated at 15°C for 15 min. The final concentrations of EDTA and lysozyme were 6.0×10^{-3} M and 0.4 mg/ml, respectively, when culture BIII 45 was used, and 1.2×10^{-2} M and 0.8 mg/ml, respectively, when BIII 82 was used.

After 15 min, the cell suspension was centrifuged at 12,000 × *g* for 10 min at 4°C. The resulting supernatant ("periplasmic") fraction was stored at 4°C, and the pellet of spheroplasts was lysed by resuspension in 40.0 ml of 0.114 M $NaHCO_3$ buffer containing 5.0 mg of deoxyribonuclease (Sigma Chemical Co.). The spheroplast suspension was incubated at 15°C with occasional shaking until noticeable clearing occurred (approximately 30 to 60 min). The lysed spheroplast suspension was first centrifuged at 1,900 × *g* for 5 min at 4°C, the resulting pellet being discarded, and then at 45,900 × *g* for 20 min at 4°C. The resulting supernatant ("intracellular") fraction was stored at 4°C whereas the pellet (membrane fraction) was washed two times in 40.0 ml of NaCl-$NaHCO_3$ solution (9.0 volumes of 3% NaCl and 1.0 volume of 0.114 M $NaHCO_3$ buffer) at 45,900 × *g* for 20 min at 4°C. The final pellet was then resuspended in 6.0 to 12.0 ml of NaCl-$NaHCO_3$ solution, unless otherwise noted.

If a starved cell suspension, prepared as described above, was to be treated with EDTA-lysozyme, it was centrifuged at 12,000 × *g* for 10 min at 4°C and the resulting pellet was resuspended in 15.0 ml of 3.0×10^{-2} M Tris buffer. The procedure from this point on was as for unstarved cells.

Difference spectroscopy. A Shimadzu-Bausch and Lomb Spectronic 200 UV, split-beam, scanning spectrophotometer, equipped with a Houston Instruments Omnigraphic X-Y recorder, was used in this study. Difference spectra were recorded at room temperature or at 15°C. Temperature control at 15°C was maintained by use of a constant-temperature cuvette holder, which was attached to a Forma Temp Junior Bath and Circulator.

The total cytochrome complement of a particular preparation was determined by standard spectrophotometric techniques (1). Complete oxidation in the reference cuvette was achieved by addition of 1 drop of 3.0% H_2O_2 in 3% NaCl, while a drop of 3.0% NaCl was simultaneously added to the sample cuvette. Cytochrome reduction in the sample cuvette was achieved by addition of a few crystals of sodium dithionite.

Carbon monoxide-binding pigments in various cell fractions were detected in 2.5-ml samples. Introduction of CO into dithionite-reduced samples was done in a Thunberg cuvette in dim light by three successive evacuations and gassings.

To study reduction of cytochromes in the electron transport particles by Mn(II), a special procedure had to be devised for the application of difference spectroscopy. This was necessary because the reaction mixture required the presence of an insoluble, opaque component, namely, MnO_2. The reaction mixture contained the following: MnO_2, 10 mg; membrane fraction, 3.9 ml; support solution (0.2% Noble agar, Difco), 3.0 ml; 0.114 M $NaHCO_3$ (pH 7.5), 0.6 ml. All components were prechilled to 15°C. The support solution was used to keep the particulate MnO_2 suspended in the cuvettes. The agar solution was made up in 3.0% NaCl and preincubated at 15°C for 16 h. A 2.6-ml amount of the reaction mixture was pipetted into each of two cuvettes and their content was examined spectrophotometrically at 15°C as follows. First, an air-oxidized minus air-oxidized spectrum was scanned (base line). Then, after adding 0.1 ml of 3×10^{-2} M NaN_3 in 3% NaCl to the sample cuvette and 0.1 ml of 3% NaCl to the reference cuvette, repeated spectra were scanned at 10-min intervals until no further changes in peak heights were observed (endogenous activity). Then, 0.3 ml of 3.5×10^{-2} M $MnSO_4$ solution was added to each of the two cuvettes, and repeated scans were made at 15-min intervals [Mn(II)-oxidizing activity]. The final concentrations of $MnSO_4$ and NaN_3 in the cuvettes were 3.5×10^{-3} and 1.0×10^{-3} M, respectively. By adding azide only to the sample cuvette while adding $MnSO_4$ to both cuvettes, we were able to overcome a problem with a slight precipitate formation that occurred when the $MnSO_4$ was added to the reaction mixture. That is, by adding $MnSO_4$ to both cuvettes, the turbidity effect from the slight precipitation in both cuvettes was cancelled out.

Identification of protohemochromogens and hemochromogens. Sonic extracts of starved cells of

culture BIII 45 and unstarved cells of culture BIII 82, grown, harvested, and washed as previously described, were prepared in 5.0 ml of 3% NaCl solution. After centrifugation of the sonic extracts at 12,000 × *g* for 10 min at 4°C, the respective supernatants were lyophilized, after determining their protein content, and extracted as described by Jacobs and Wolin (7). Spectra of the chromogens were measured in pyridine-KOH, as described by the same authors.

Protein determination. The protein concentration of the various cell fractions was determined by using the method of Lowry et al. (11) as modified by Henry (6). The total protein content in the periplasmic and intracellular fractions was corrected for the presence of lysozyme or deoxyribonuclease, respectively.

RESULTS

Cytochrome complements of various fractions from cultures BIII 45 and BIII 82. The dithionite-reduced minus air-oxidized difference spectra of the membrane preparations from starved culture BIII 45 and unstarved culture BIII 82 (Fig. 1A and 2A) indicated the presence of type *b* and *c* cytochromes characterized by α peaks at 561 and 554 to 555 nm, respectively, and β peaks at 532 and 525 to 526 nm, respectively. These spectra showed no peaks corresponding to type *a* cytochrome. The γ peak at 429 nm is best interpreted as a combined peak of the type *b* and *c* cytochromes because of its

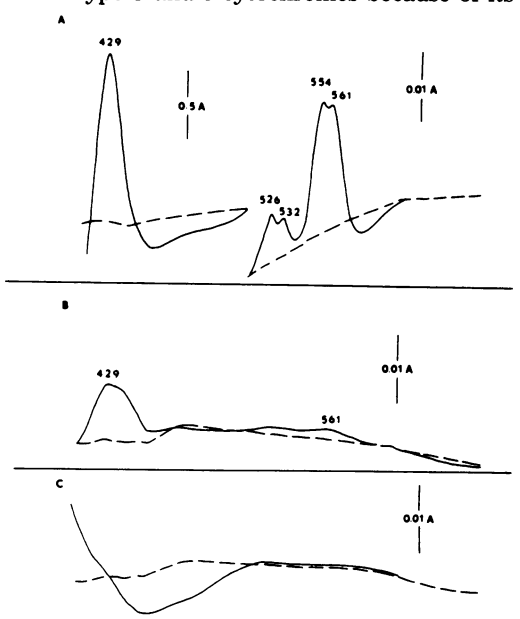

FIG. 1. *Air-oxidized minus air-oxidized (- - -) and dithionite-reduced minus air-oxidized (——) difference spectra of the membrane (A), periplasmic (B), and intracellular (C) fractions of culture BIII 45. The total protein in each cuvette was 16.5, 9.0, and 13.2 mg for the membrane, periplasmic, and intracellular fractions, respectively.*

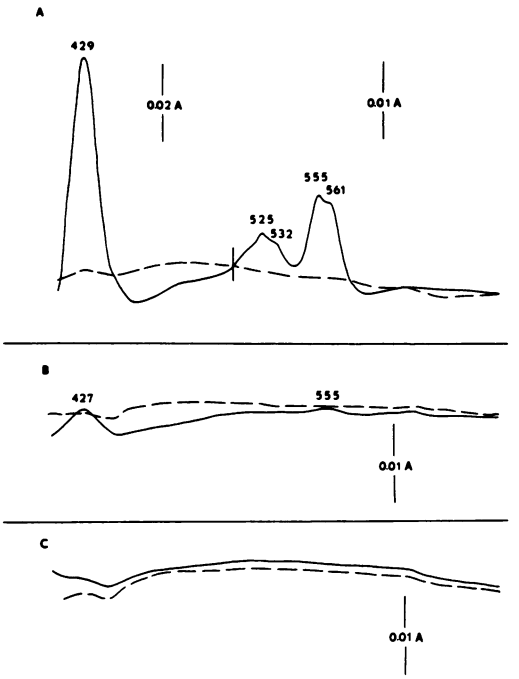

FIG. 2. *Air-oxidized minus air-oxidized (- - -) and dithionite-reduced minus air-oxidized (——) difference spectra of the membrane (A), periplasmic (B), and intracellular (C) fractions of culture BIII 82. The total protein in each cuvette was 10.50, 5.10, and 5.32 mg for the membrane, periplasmic, and intracellular fractions, respectively.*

asymmetry and broadness. The periplasmic fractions of the two cultures showed broad peaks at 555, 561, 427, and 429 nm, which probably resulted from the combined absorbance of type *b* and *c* cytochromes (Fig. 1B and 2B). The intracellular fractions from both strains showed no evidence of cytochromes (Fig. 1C and 2C).

The identity of the type *b* and *c* cytochromes in the two strains was confirmed by demonstration of ether-soluble pyridine protohemochromogen with an α peak at 557 to 558 nm and acetone-HCl-insoluble pyridine hemochromogen with an α peak at 552 nm.

The CO-dithionite-reduced minus dithionite-reduced difference spectra of membrane and periplasmic fractions from starved cells of culture BIII 45 and from unstarved culture BIII 82 indicate the presence of cytochrome *o*, a type *b* cytochrome, as evidenced by peaks at 568 to 571, 546 to 554, and 417 to 424 nm (Fig. 3 and 4).

Cytochrome reduction coupled to Mn(II) oxidation by a combination of cell fractions of culture BIII 45. Since the membrane and periplasmic fractions of starved cells of culture BIII 45 contained significant amounts of cyto-

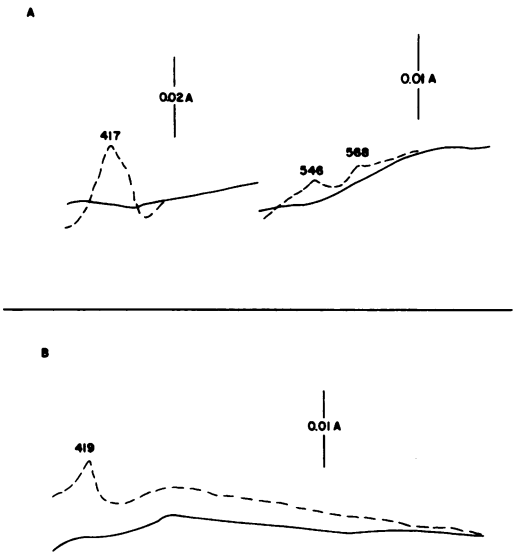

FIG. 3. *Dithionite-reduced minus dithionite-reduced (——) and CO-dithionite-reduced minus dithionite-reduced (- - -) difference spectra of the membrane (A) and periplasmic (B) fractions from a starved cell suspension of culture BIII 45. The total protein in each cuvette was 24.8 and 5.3 mg for the membrane and periplasmic fractions, respectively.*

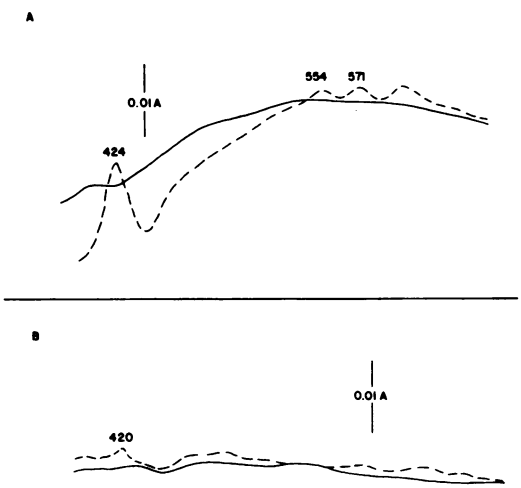

FIG. 4. *Dithionite-reduced minus dithionite-reduced (——) and CO-dithionite-reduced minus dithionite-reduced (- - -) difference spectra of the membrane (A) and periplasmic (B) fractions from culture BIII 82. The total protein in each cuvette was 15.0 and 13.8 mg for the membrane and periplasmic fractions, respectively.*

chromes, they were tested individually by difference spectroscopy for their ability to couple cytochrome reduction to Mn(II) oxidation. In the case of the periplasmic fraction, neither the addition of azide nor the subsequent addition of Mn(II) resulted in reduction of its cytochrome complement. In the case of the membrane fraction, suspended in 12.0 ml of NaCl-$NaHCO_3$ solution, the addition of azide resulted in peak formation characteristic of a type *c* cytochrome and, possibly, a type *b* cytochrome. Since no exogenous reductant had been added up to this point, this reduction must be attributed to the action of an endogenous electron donor, whose electrons were at least partially prevented by azide from passing to oxygen, thereby causing progressive reduction of the cytochromes behind the azide block. The subsequent addition of Mn(II) resulted in varied responses in different experiments. In certain instances, addition of Mn(II) caused a slight increase in peak height of the already existing type *c* cytochrome peaks formed after azide addition, indicating the reduction of type *c* cytochrome by Mn(II). Yet, in other experiments, the addition of Mn(II) was without effect.

When, however, the membrane fraction was suspended in 12.0 ml of the periplasmic fraction instead of the NaCl-$NaHCO_3$ solution, the results shown in Fig. 5 were consistently observed. The addition of azide to the sample cuvette caused the emergence of α and γ peaks of type *c* cytochrome at 554 and 425 nm, respectively, due to reduction by an endogenous electron donor. These peaks reached their maximum height within 10 min after the addition of azide. When, after 20 min, Mn(II) was added to both cuvettes and the resulting difference spectra were scanned at timed intervals, further changes in the spectrum were noted. The difference spectrum obtained 75 min after addition of Mn(II), shown in Fig. 5, revealed a definite increase in both the peak at 425 nm and that at 554 nm. The increase at 425 nm after the addition of

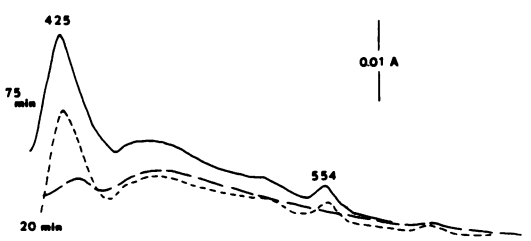

FIG. 5. *Difference spectra of the combined membrane and periplasmic fractions of starved cells of culture BIII 45. Symbols: (— —) air-oxidized minus air-oxidized spectrum; (·····) difference spectrum 20 min after NaN_3 was added to the sample cuvette to a final concentration of 10^{-3} M; (——) difference spectrum 75 min after $MnSO_4$ was added to both cuvettes to a final concentration of 3.5×10^{-3} M. The total protein in each cuvette was 13.67 mg.*

Mn(II) is approximately 69% over the absorbance seen at 425 nm before the addition of Mn(II). The percent increase was calculated as follows: {[(A_{425} − A_{470}) after Mn(II) addition − (A_{425} − A_{470}) before Mn(II) addition]/[(A_{425} − A_{470}) before Mn(II) addition]} × 100, where A_{425} is the absorbance at 425 nm and A_{470} is the absorbance at 470 nm. The increase seen at 425 nm after the addition of Mn(II) also represents approximately 7.0% of the total A_{425}, i.e., when the cytochrome was reduced completely. Total A_{425} due to complete cytochrome reduction was measured in a dithionite-reduced minus hydrogen peroxide-oxidized difference spectrum of a separate sample of the membrane preparation. In other similar experiments, the addition of Mn(II) caused increases in A_{425} as great as 100%.

Thus, it appears that one or more factors in the periplasmic fraction of culture BIII 45 were complementing the membrane fraction in some manner which enabled the reduction of cytochrome by Mn(II). The intracellular fraction did not contribute complementing factors to the membrane fraction, as was shown in an experiment in which membrane fraction was resuspended in 12.0 ml of the intracellular fraction. When azide was added to the sample cuvette in this instance, peaks characteristic of the reduction of a type *c* cytochrome appeared, but when Mn(II) was subsequently added to both cuvettes, no enhancement of the type *c* cytochrome peaks occurred. In fact, the peaks decreased slightly with time.

Neither the EDTA nor the lysozyme contained in the periplasmic fraction was responsible for the enhancement in cytochrome reduction seen upon Mn(II) addition to the membrane fraction which had been resuspended in the periplasmic fraction. This was shown in an experiment in which the membrane fraction was resuspended in 12.0 ml of Tris buffer-EDTA-lysozyme solution (15 ml of 3.0×10^{-2} M Tris plus 5.0 ml of 3.0×10^{-2} M EDTA plus 5.0 ml of a 2.0-mg/ml solution of lysozyme in 3.0% NaCl). Whereas the addition of azide to the sample cuvette produced peaks characteristic of the reduction of type *c* cytochrome, the subsequent addition of Mn(II) did not result in the enhancement of the type *c* cytochrome peaks.

The requirement of MnO_2 for cytochrome reduction by Mn(II) was shown in still another experiment in which the membrane fraction of culture BIII 45 resuspended in the periplasmic fraction was used in a reaction mixture from which MnO_2 was omitted. In this instance, the addition of azide resulted in the production of peaks characteristic of the reduction of a type *c* cytochrome. However, subsequent addition of Mn(II) did not cause further increase in the peak height of the type *c* cytochrome, as was expected, since it has been shown that the Mn(II) must first be bound to MnO_2 before it can be oxidized by this culture (3).

Cytochrome reduction coupled to Mn(II) oxidation by a combination of cell fractions of culture BIII 82. The membrane fraction of culture BIII 82, suspended in NaCl-$NaHCO_3$ solution, gave results qualitatively similar to those with membranes from culture BIII 45 upon addition of azide. Upon subsequent addition of Mn(II), no enhancement of already existing peaks was ever noted. When the membrane fraction of culture BIII 82 was suspended in 12.0 ml of periplasmic fraction, the addition of azide and subsequently Mn(II) yielded responses qualitatively similar to those with corresponding preparations from culture BIII 45 (Fig. 6). The azide addition caused production of α and γ peaks of type *c* cytochrome at 553 and 425 nm, respectively. The subsequent addition of Mn(II) caused enhancement of the α and γ peaks of the type *c* cytochrome. The increase in A_{425} after addition of Mn(II) was approximately 56% over that before the addition of Mn(II), and also approximately 8.0% of that due to total reduction of the type *c* cytochrome. Thus, it again appeared that a factor(s) in the periplasmic fraction was complementing the membrane fraction in such a way as to allow the type *c* cytochrome of the membrane fraction to be reduced at the expense of the Mn(II) oxidation.

In another experiment, the membrane fraction of culture BIII 82 was suspended in 12.0 ml of the intracellular fraction. As the results in Fig. 7 show, the addition of azide caused the production of α and γ peaks of type *c* cytochrome at 553 and 424 nm, respectively. Subsequent addition of Mn(II) caused an enhancement of these peaks. The increase in A_{424} after the addition of Mn(II) was approximately 50% over that before the addition of Mn(II) and approximately 35% of the total A_{424} when the type *c* cytochrome

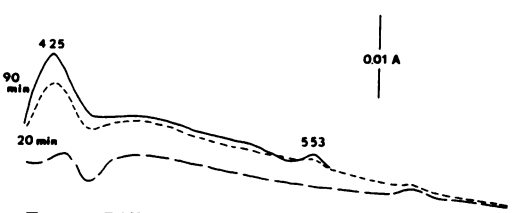

FIG. 6. *Difference spectra of the combined membrane and periplasmic fractions of culture BIII 82. Symbols: (— —) air-oxidized minus air oxidized spectrum; (-----) difference spectrum 20 min after NaN_3 was added to the sample cuvette; (——) difference spectrum 90 min after $MnSO_4$ was added to both cuvettes. The total protein in each cuvette was 13.69 mg.*

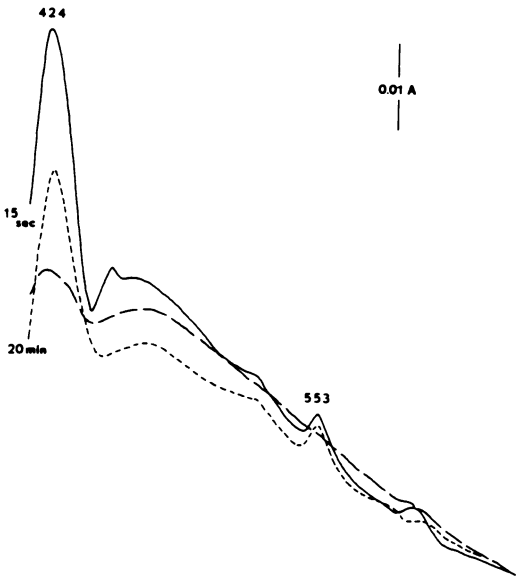

FIG. 7. *Difference spectra of the combined membrane and intracellular fractions of culture BIII 82. Symbols: (— —) air-oxidized minus air-oxidized spectrum; (----) difference spectrum 20 min after NaN_3 was added to the sample cuvette; (——) difference spectrum 15 s after $MnSO_4$ was added to both cuvettes. The total protein in each cuvette was 18.28 mg.*

was completely reduced. Thus, it appears that one or more constituents of the intracellular fraction of culture BIII 82 can complement its membrane fraction to allow for the reduction of a type *c* cytochrome at the expense of Mn(II) oxidation. These findings suggest the possibility that the activity of the periplasmic fraction may be attributable to contamination by intracellular components due to premature lysis of some spheroplasts during the EDTA-lysozyme treatment of the cells.

The deoxyribonuclease contained in the intracellular fraction was not responsible for the enhancement in cytochrome reduction seen upon Mn(II) addition to the membrane fraction which had been resuspended in the intracellular fraction. This was shown in an experiment in which the membrane fraction was resuspended in 12.0 ml of $NaHCO_3$ buffer containing approximately 5.0 mg of deoxyribonuclease. The subsequent addition of azide to the sample cuvette produced peaks characteristic of the reduction of a type *c* cytochrome, but the subsequent addition of Mn(II) to both cuvettes did not result in the enhancement of the type *c* cytochrome peaks.

When membrane fraction of culture BIII 82 resuspended in the intracellular fraction was used in the same experiment as above, except that MnO_2 was omitted from the reaction mixture, the addition of azide again resulted in the production of peaks characteristic of the reduction of a type *c* cytochrome. However, the subsequent addition of Mn(II) did not cause a further increase in the peak height of the type *c* cytochrome, once more demonstrating a requirement for MnO_2 in the reaction mixture for Mn(II) to be oxidized.

DISCUSSION

Cultures BIII 45 and BIII 82, two Mn(II)-oxidizing bacteria, have been found to contain only type *b* and *c* cytochromes and no type *a* cytochromes when grown under the conditions of the experiments reported here. These results should not be taken to imply that Mn(II)-oxidizing organisms never contain type *a* cytochromes. Further work, to be reported elsewhere, has shown the presence of a type *a* cytochrome, cytochrome aa_3, in three other Mn(II)-oxidizing bacteria, two of which were grown under the same conditions as reported in this work.

The present study provides evidence for the presence of cytochrome *o* in both cultures BIII 45 and BIII 82. Since no evidence for a type *a* cytochrome was found, and since both cultures exhibit reduced nicotinamide adenine dinucleotide dehydrogenase activity (data not shown), cytochrome *o* probably functions as the cytochrome oxidase in both cultures. A presence of type *b*, *c*, and *o*-cytochromes, and an absence of type *a* cytochrome, in certain spirilla has been previously noted (8).

Both cultures BIII 45 and BIII 82 were shown to be able to couple Mn(II) oxidation reproducibly to the reduction of a type *c* cytochrome. However, the conditions under which this coupling occurred varied considerably between the two organisms. In the case of culture BIII 45, the cells had to be starved before fractionation to eliminate a strong, nonspecific cytochrome reduction by endogenous oxidizable substrate, which was otherwise observed when azide was added to the sample cuvette containing the various fractions from unstarved cells of culture BIII 45. With unstarved cells, spectral scanning after addition of azide to the sample cuvette initially revealed characteristic peaks which decreased in height with time. This decrease in peak height was due to more rapid oxygen consumption in the reference cuvette to which azide, which blocks oxygen consumption, had not been added. On longer incubation (20 min), the difference spectrum of the cytochromes became inverted because the contents of the reference cuvette had now become anaerobic, whereas the contents of the sample cuvette, containing azide, remained aerobic. A stable difference spectrum

was never obtained with unstarved cells of culture BIII 45 in a reasonable amount of time. Therefore, it was impossible to observe enhancement in cytochrome reduction upon subsequent addition of Mn(II) to the cuvettes. Cell extracts from starved cells of culture BIII 45 did not present this problem when used in similar studies. Starvation of culture BIII 82 was not necessary before fractionation because the strong interference by endogenous substrate seen in the case of culture BIII 45 was not noted.

Cultures BIII 45 and BIII 82 also differed in these experiments in that the membrane fraction prepared from culture BIII 45 could only couple cytochrome *c* reduction to Mn(II) oxidation reproducibly when that fraction was suspended in the periplasmic fraction, whereas the membrane fraction of culture BIII 82 could couple cytochrome *c* reduction to Mn(II) oxidation when that fraction was suspended in either the periplasmic or the intracellular fraction. This observed difference could reflect a difference in the mechanisms involved in the Mn(II) oxidation by the two organisms or a difference in the cellular distribution of the unsedimented active factor. The separation of the Mn(II)-oxidizing enzyme system of cultures BIII 45 and BIII 82 into a membrane fraction and a nonsedimented fraction which have to be combined for observing Mn(II)-oxidizing activity has been confirmed, using the persulfate assay (3) for the enzyme activity. Details of these results will be presented in another communication.

It might be argued that the Mn(II) ion itself was not causing the reduction of type *c* cytochrome in culture BIII 45 or BIII 82 but was merely stimulating the cytochrome reduction by some endogenous substrate, which resulted in the enhancement of the observed reduction of the type *c* cytochrome. This is not a likely explanation because in order to see enhancement in reduction of the type *c* cytochrome by Mn(II), insoluble MnO_2 had to be included in the reaction mixture. If the Mn(II) had just acted as a cofactor, the presence or absence of MnO_2 should not have mattered.

Finally, the question may be raised why only a type *c* cytochrome in cultures BIII 45 and BIII 82 appeared to be distinctly reduced by Mn(II) in these spectrophotometric studies. Since the electron transport system in these experiments was blocked at the level of cytochrome oxidase, and since antimycin A and 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide have previously been shown to inhibit Mn(II) oxidation in cultures BIII 45 (5) and BIII 82 (Ehrlich, unpublished data), a type *b* cytochrome would be expected to be reduced by Mn(II) in addition to cytochrome *c*. This discrepancy can be explained by the incomplete blockage of electron transport by azide in the present experiments, which merely resulted in a shift in the steady-state ratio of reduced to oxidized cytochrome toward the reduced state instead of its complete reduction. The incomplete blockage of electron transport by azide was inferred from experiments with reduced nicotinamide adenine dinucleotide (data not shown) in which only temporary reduction of cytochromes was achieved in the presence of azide. The lack of peaks corresponding to reduced type *b* cytochrome could thus have been the result of an Mn(II)-induced shift in steady-state ratio of reduced to oxidized cytochromes which was not great enough to bring about sufficient build-up of reduced type *b* cytochrome to be spectrophotometrically detectable, even though this cytochrome appears to be involved in the electron transport system coupled to Mn(II) oxidation. If azide had blocked electron transport in the two cultures completely, enhancement of type *c* cytochrome reduction by Mn(II) would not have been observed if the oxidizable endogenous substrate in the preparations had reduced the cytochromes completely.

We must also ask why absorption peaks characteristic of the type *o* cytochrome, which appears to be the only cytochrome oxidase present in these organisms, do not appear in the Mn(II)-reduced difference spectra. A probable explanation for this could be that inhibition caused by azide is the result of the binding of the azide to the oxidized form of cytochrome *o*. Azide is known to bind to the oxidized form of hemoprotein such as cytochrome a_3 (10).

The involvement of cytochromes in the oxidation of Mn(II) provides further evidence that the Mn(II)-oxidizing bacteria may derive useful energy from the oxidation of Mn(II) by oxidative phosphorylation using a conventional electron transport chain, as suggested by the findings of Ehrlich (5).

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