

Effects of Seawater Cations and Temperature on Manganese Dioxide-Reductase Activity in a Marine *Bacillus*

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The seawater cations, Na⁺, K⁺, Mg²⁺, and Ca²⁺, each stimulated MnO₂-reductase activity of whole cells and cell extracts of *Bacillus* 29. Concentrations of Na⁺ and K⁺ which stimulated whole cells and cell extracts maximally were equivalent to those in two- to fivefold diluted seawater. Cell-extract activity was strongly stimulated by Ca²⁺ and Mg²⁺ up to a concentration of 0.01 M Mg²⁺ and 0.002 M Ca²⁺, with little additional stimulation above these concentrations. Whole-cell activity was stimulated biphasically with increasing concentrations of Ca²⁺ and Mg²⁺. Comparison of the effects of individual cations or mixtures of them at concentrations equivalent to their concentration in fivefold diluted seawater showed that more activity was obtained with 0.01 M Mg²⁺ or 0.002 M Ca²⁺ than with 0.1 M Na⁺, and more with 0.1 M Na⁺ than with 0.002 M K⁺. Fivefold diluted seawater permitted as much or more activity as solutions of individual or synthetic mixtures of the cations. Pre-exposure experiments showed that the ionic history of whole cells was important to their ultimate activity. The MnO₂-reductase activity of induced whole cells exhibited a temperature optimum near 40 C. Cell extracts had different temperature optima (T_{opt}), depending on whether induced glucose-linked activity (T_{opt} = 25 C), uninduced glucose-linked, ferricyanide-dependent activity (T_{opt} = 30 C), or uninduced ferrocyanide-linked activity (T_{opt} = 40 C) were being measured. Some of these optima are higher than previously reported.

The participation of bacteria in a marine manganese cycle was established by studying the bacteriology of marine manganese nodules (2-5; 8, 15, 16). Manganese nodules yielded bacteria which participate in both oxidation and reduction of manganese (2, 4, 6). The enzymatic nature of these reactions has been demonstrated (4, 5, 16). The biochemistry of the enzymatic interactions with manganese was also investigated in the bacteria isolated from nodules, and characterization of the enzymes involved has begun (5; 8; 16; Ghiorse and Ehrlich, *Annu. Meet., Amer. Soc. Microbiol., Philadelphia, Pa., Abstr. 163, p. 163, 1972*).

Since bacterial reduction of Mn(IV) in nodules on the ocean floor must occur in a saline environment, it is important to determine what influence, if any, the major cations of seawater (Na⁺, K⁺, Mg²⁺, and Ca²⁺) have on the process. Similarly, since bacterial Mn(IV) reduction on the ocean floor must occur at a low temperature, it is of interest to determine what effect various temperatures have on this process. This report deals with these effects on MnO₂ reduction by

Bacillus 29, which was isolated from a manganese nodule from Blake Plateau in the Atlantic Ocean and shown to produce an inducible MnO₂-reductase system (15, 16).

(The data in this paper were taken from a thesis submitted by W. C. Ghiorse to the Department of Biology of Rensselaer Polytechnic Institute in partial fulfillment of the requirements for the Ph.D. degree).

MATERIALS AND METHODS

Bacterial cultures. *Bacillus* 29 cultures (2) were maintained at 25 C on Stock Culture agar (Difco). For experimentation, uninduced cells (i.e., cells which required the electron carrier, ferricyanide, for MnO₂-reduction) were grown on seawater nutrient-agar in Roux slants as described previously (16). Induced cells (i.e., those which did not require ferricyanide for MnO₂-reduction) were obtained by growth in the presence of Mn²⁺. This was accomplished by adding 1 ml of sterile 0.1 M MnSO₄·H₂O to 9 ml of inoculum suspended in seawater and distributing the mixture over the seawater nutrient-agar surface. These cultures were incubated aerobically at 25 C for 24 h.

Preparation of whole cell suspension. Whole cells of *Bacillus* 29 were removed from 1 to 12 Roux slants with seawater. The resultant suspension was cen-

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trifuged at $10,000 \times g$ for 10 min. Uninduced cells were then washed two or three times in distilled water and 1-ml samples of the final distilled-water suspension was then used to inoculate the various reaction mixtures. Induced cells were washed in distilled water or in fivefold diluted seawater, depending on the experiment, until 9.0 ml of the wash solution was free of Mn^{2+} as indicated by persulfate oxidation. Washed whole cells were then suspended in an appropriate medium and either assayed directly or ruptured by sonication to produce cell-free extracts.

Preparations of cell-free extracts. Cells of *Bacillus* 29 which had been washed three times in distilled water were suspended in 5 to 15 ml of appropriate seawater or salts solution to a final cell concentration of 2 to 4% (dry cell weight per suspension volume) and sonicated for 4 to 6 min according to the method of Trimble and Ehrlich (16). Sonic extracts were centrifuged at $15,000 \times g$ for 15 min at 4 C. Supernatants containing the enzymatic activity were free of cell debris when examined by phase contrast microscopy.

Assays for MnO_2 -reductase activity. Two assays for MnO_2 -reductase activity were used. Both were modifications of the short-term assay of Trimble and Ehrlich (15). In one modification, the glucose-linked activity of either ferricyanide-dependent or ferricyanide-independent preparations was measured in duplicate 50-ml Erlenmeyer flasks containing 0.2 g of MnO_2 (prepared as described by Trimble and Ehrlich, 15), 5.0 ml of appropriate seawater or salts solution, 1.0 ml of 1% glucose dissolved in appropriate seawater or salts solution, and 1.0 ml of either whole-cell suspension or cell-free extract. In the case of uninduced cell preparations, 0.1 ml of 0.01 M $K_3Fe(CN)_6$ was also added to the flasks. In the second assay modification, the ferrocyanide-linked activity of uninduced preparations was measured by altering the glucose-linked, ferricyanide-dependent assay in the following ways: (i) 1.0 ml of appropriate seawater or salts solution replaced the 1% glucose solution; (ii) 0.1 ml of 0.04 M $K_4Fe(CN)_6 \cdot 6H_2O$ or $Na_4Fe(CN)_6 \cdot 10H_2O$ replaced the 0.01 M $K_3Fe(CN)_6$. Both assay mixtures were incubated for 3 h. After incubation, the reaction mixtures were acidified by adding 0.05 ml of 10 N H_2SO_4 to each and incubating for 10 min at room temperature. The liquid portion in each reaction vessel was then centrifuged at $4,000 \times g$ for 10 min to remove any suspended MnO_2 . The amounts of manganese in the supernatants were determined by the persulfate oxidation method of Ehrlich (2), except that 1.0-ml portions of the samples were added to tubes which contained 8.0 ml of distilled water and 1.0 ml of "special reagent". In all experiments suitable controls were assayed along with experimental reaction mixtures. The difference in the amount of manganese released with and without cells or cell extract was taken as a measure of the amount of manganese released enzymatically. This difference was used to compute the specific activity of whole cells and cell extracts expressed as nanomoles of Mn^{2+} released per hour per milligram of protein. The activity was linear over the 3-h incubation period.

Estimation of protein content. Protein content of

cell extracts was estimated by the method of Lowry et al. (11), using bovine serum albumin as a standard. For whole cells of *Bacillus* 29, the average percentage of protein per cell (dry weight) was found to be $56 \pm 2\%$, based on more than 30 determinations. This value was used to estimate the amount of protein added to reaction flasks when specific whole-cell activity was measured.

Determinations of the effects of the major cations of seawater on MnO_2 -reductase activity. Cells and cell-free extracts were tested in reaction mixtures containing one or more of the chloride salts of Na, K, Mg, or Ca at concentrations up to and beyond those in natural seawater. Reaction mixtures containing uninduced cells were incubated for 3 h at 25 C and those containing induced cells were incubated for 4.5 h at 40 C. After incubation, the contents of the flasks were acidified with 0.05 ml of 10 N H_2SO_4 for 10 min. After acidification, the liquid portion in each reaction vessel was clarified by centrifugation and the supernatant was assayed for the manganese released.

Determination of the effects of pre-exposing, uninduced whole cells to various cation solutions. For these experiments, uninduced cells were washed twice in distilled water then resuspended in either distilled water, 0.1 M NaCl, 0.059 M $MgCl_2 \cdot 6H_2O$, or 0.059 M CaCl₂. Measured portions of resultant suspensions were then transferred to appropriate reaction mixtures, giving a final cation concentration as specified, and assayed for glucose-linked, ferricyanide-dependent MnO_2 -reductase activity. After incubation at 25 C for 3 h, the contents of each flask were acidified, clarified by centrifugation, and assayed for released manganese.

Determination of the effect of temperature on MnO_2 -reductase activity. Uninduced whole cells were washed and suspended in the medium in which they were tested (fivefold diluted seawater, 0.05 M $MgCl_2 \cdot 6H_2O$ or 0.1 M NaCl). Preparations of induced cells were washed and suspended in fivefold diluted seawater and tested in a reaction mixture made up in that solution. Reaction mixtures were incubated at temperatures between 10 and 60 C. Those held at temperatures between 10 and 25 C were incubated in low-temperature incubators. Those subjected to temperatures between 28 and 60 C were incubated in thermostatically controlled water baths. Uninduced cell preparations were incubated for 3 h and induced cell preparations for 4.5 h. The temperature of all reaction mixtures was permitted to reach that of the room before acidification. Inoculated mixtures were incubated at the same time as blank controls for each experimental temperature point above 25 C since an increase in nonenzymatic release of Mn^{2+} was observed in mixtures incubated at 30 C and above. Nonenzymatic release of Mn^{2+} in reaction mixtures incubated at temperatures below 25 C was considered no greater than at 25 C. Therefore, the 25 C control was used as an estimate of the amount of Mn^{2+} released in mixtures incubated at the lower temperatures. Since the number of experimental temperatures of incubation which could be tested in a single experiment was limited, results from several experi-

ments in which cells were incubated at different temperatures were pooled to study the effect of temperature range on a given process.

RESULTS

Effects of Na^+ , K^+ , Mg^{2+} , and Ca^{2+} on MnO_2 -reductase activity. The glucose-linked, ferricyanide-dependent, and the ferrocyanide-linked MnO_2 -reductase activities of uninduced *Bacillus* 29 were stimulated by individual increases in concentration of each of the four major cations of seawater, Na^+ , K^+ , Mg^{2+} , and Ca^{2+} (Fig. 1-3). Na^+ and K^+ exerted a qualita-

tively similar effect on the glucose-linked activity of whole cells (Fig. 1), cell extracts (Fig. 2), and the ferrocyanide-linked activity of cell extracts (Fig. 3). Greatest stimulation was demonstrated in reaction mixtures containing concentrations of the monovalent cations that would be found in two- to fivefold diluted seawater. Mg^{2+} and Ca^{2+} , on the other hand, exerted dissimilar effects on whole-cell and cell-extract activities. The activity of whole cells was stimulated in a biphasic manner, being greatest in the assay mixtures containing the highest Mg^{2+} and

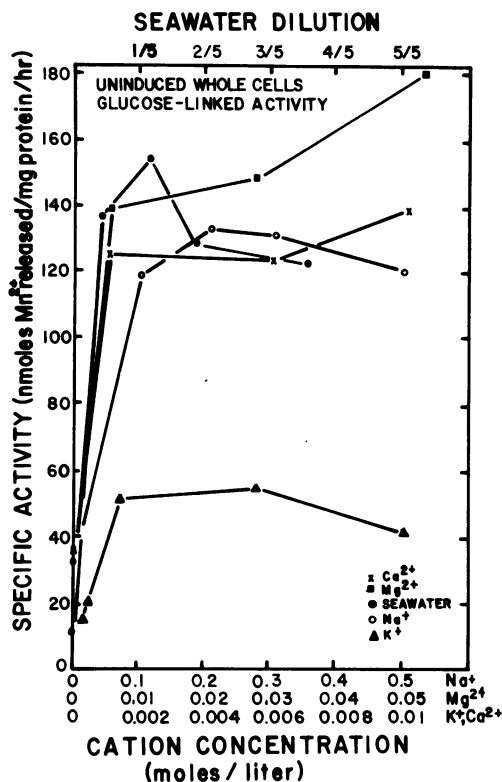


FIG. 1. Effects of various cation concentrations and seawater dilutions on glucose-linked, ferricyanide-dependent MnO_2 -reductase activity in uninduced whole cells of *Bacillus* 29. For the tests with Na^+ , the cells were washed two times in distilled water for one experiment and three times for a second experiment without a significant difference in results. For experiments with the other cations and seawater dilutions, the cells were always washed three times in distilled water. The cells were added in a distilled water suspension to each reaction mixture. The final cation concentration of each reaction mixture was as specified. Each reaction mixture contained 0.0004 M K^+ from $K_3Fe(CN)_6$, which probably accounts for minimal activity in the absence of other added salts.

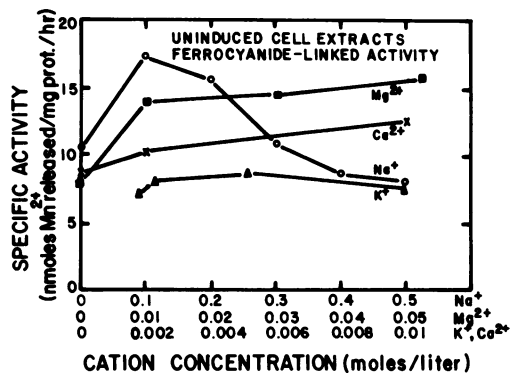


FIG. 2. Effects of various cation concentrations and seawater dilutions on glucose-linked, ferricyanide-dependent MnO_2 -reductase activity in extracts of uninduced cells of *Bacillus* 29. The cells were washed three times in distilled water and sonicated in distilled water. Each reaction mixture contained 0.0004 M K^+ from $K_3Fe(CN)_6$, which probably accounts for minimal activity in the absence of other added salts.

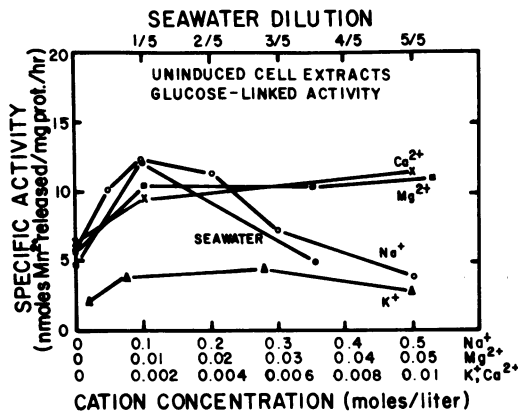


FIG. 3. Effects of various cation concentrations of ferrocyanide-linked MnO_2 -reductase activity in extracts of uninduced cells of *Bacillus* 29. Extracts were prepared as for experiments in Fig. 2. Each reaction mixture contained 0.0022 M K^+ from $K_3Fe(CN)_6 \cdot 3H_2O$, which probably accounts for minimal activity in the absence of other salts.

Ca²⁺ concentrations tested (Fig. 1, Table 1); whereas both activities in cell extracts were stimulated strongly only up to approximately 0.01 M Mg²⁺ and 0.002 M Ca²⁺ (Fig. 2 and 3). Further increases in the concentration of Ca²⁺ and Mg²⁺ resulted in only minor stimulation.

The molar concentrations of the four major cations in seawater are approximately as follows: Na⁺, 0.5 M; Mg²⁺, 0.05 M; Ca²⁺, 0.01 M; and K⁺, 0.01 M. At concentrations corresponding to those in fivefold diluted seawater, 0.01 M Mg²⁺ or 0.002 M Ca²⁺ in the presence of 0.0022 M K⁺ supported greater activity of uninduced whole cells and cell extracts than 0.1 M Na⁺; and 0.1 M Na⁺ in the presence of 0.0022 M K⁺ supported greater activity than 0.0022 M K⁺ alone (Table 1). In the absence of other cations, 0.01 M Mg²⁺ also supported greater activity than 0.1 M Na⁺ alone. Indeed, the activity of induced cells was greatest in reaction mixtures containing the highest Mg²⁺ concentration tested (Table 2).

Mixtures of Na⁺, Mg²⁺, and K⁺, or Na⁺, Ca²⁺, and K⁺, or Na⁺, Mg²⁺, Ca²⁺, and K⁺, with each cation at a concentration equivalent to that in fivefold diluted seawater, supported activity in uninduced whole cells and extracts at about the same level as a mixture containing 0.01 M Mg²⁺ plus 0.0022 M K⁺ exhibited. The only exception was the ferrocyanide-linked ac-

tivity of extracts in mixtures containing Na⁺, Mg²⁺, and K⁺, or Na⁺, Ca²⁺, and K⁺, which was depressed for unknown reasons (Table 1).

Comparisons of these results showed that fivefold diluted seawater was the most stimulatory suspension medium for whole cells and extracts (Tables 1 and 2); the only exceptions were solutions of 0.05 M Mg²⁺ and 0.025 M

TABLE 2. Effects of various cations in solutions and in diluted seawater on MnO₂-reductase activity of whole cells of induced *Bacillus 29*^a

Reaction mixture ^b	Sp act ^c (nmol of Mn ²⁺ /h per mg of protein)
Distilled water	7.0
0.1 M Na ⁺	10.3
0.01 M Mg ²⁺	15.9
0.05 M Mg ²⁺	18.8
0.25 M Mg ²⁺	20.6
One-tenth seawater	18.0
One-fifth seawater	23.2
Six-sevenths seawater	2.2

^a Cells were washed in distilled water before incubation.

^b Chloride salts of elements yielding the appropriate cations were dissolved in distilled water.

^c Glucose-linked activity of whole cells. Reaction mixtures were incubated at 40 C, which was found to provide maximal activity (see Fig. 6).

TABLE 1. Effects of various cations in solutions and in diluted seawater on MnO₂-reductase activity of uninduced cells of *Bacillus 29*^a

Reaction mixture ^b	Sp act (nmol of Mn ²⁺ /h per mg)		
	Whole cells	Extracts	
		Glucose ^c	Glucose ^c
0.0022 M K ⁺	42.0	5.8 ± 0.3	8.3 ± 0.2
0.1 M Na ⁺ and 0.0022 M K ⁺	98.0	6.8 ± 0.1	9.4 ± 0.4
0.01 M Mg ²⁺ and 0.0022 M K ⁺	128.0	10.6 ± 0.2	13.1 ± 0.5
0.05 M Mg ²⁺ and 0.0022 M K ⁺	168.0		
0.25 M Mg ²⁺ and 0.0022 M K ⁺	186.0		
0.002 M Ca ²⁺ and 0.0022 M K ⁺	135.0		10.0 ± 0.6
0.05 M Ca ²⁺ and 0.0022 M K ⁺	177.5		
0.1 M Na ⁺ , 0.01 M Mg ²⁺ , and 0.0022 M K ⁺	146.5	11.2 ± 0.3	6.3 ± 0.1
0.1 M Na ⁺ , 0.01 M Ca ²⁺ , and 0.0022 M K ⁺			4.6 ± 0.6
0.1 M Na ⁺ , 0.01 M Mg ²⁺ , 0.002 M Ca ²⁺ , and 0.0022 M K ⁺	125.5	10.2 ± 0.5	11.4 ± 0.5
One-fifth seawater and 0.0022 M K ⁺	149.5	12.2 ± 0.5	12.0 ± 0.4
Five-sevenths seawater and 0.0022 M K ⁺	128.0		

^a Cells were washed in distilled water before incubation or sonication. Assays were performed at 25 C.

^b Chloride salts of elements yielding the indicated cations were dissolved in distilled water. The K⁺ concentration was derived from 0.0004 M K⁺ from K₃Fe(CN)₆, and from 0.0018 M K⁺ from KCl, or from 0.0022 M K⁺ from K₄Fe(CN)₆ · 3H₂O.

^c Glucose-linked, ferricyanide-dependent activity.

^d Ferrocyanide-linked activity.

Mg^{2+} with uninduced whole cells. Lesser dilutions of seawater supported lower activities (Fig. 1 and 2, Tables 1 and 2), which may be attributed to the presence of high concentrations of Na^+ and K^+ , both of which supported less activity when used in concentrations found in full-strength seawater (Fig. 1 and 2).

Experiments designed to determine the effects of pre-exposure of uninduced whole cells to solutions containing various cations (Table 3) showed that the ionic history of whole cells affected the MnO_2 -reductase activity subsequently determined. Pre-exposure to media containing 0.059 M Mg^{2+} or Ca^{2+} before assay produced higher specific activity than pre-exposure to distilled water when 0.05 M Mg^{2+} or Ca^{2+} was present in the reaction mixture (Table 3). Furthermore, the higher specific activity was not diminished when the Mg^{2+} or Ca^{2+} concentration of the reaction mixture was lowered to 0.008 M. Washing cells once with distilled water after Mg^{2+} pretreatment decreased the specific activity to about 51% of the original value obtained with 0.008 M Mg^{2+} in the reaction mixture. A second washing diminished the

activity an additional 8% (Table 3). These results indicate that Mg^{2+} , and probably Ca^{2+} , were rather tightly bound to cells. A similar retention of activity was not observed with 0.1 M Na^+ (Table 3) suggesting that this cation was loosely bound, or not bound at all, by the cells.

Effect of temperature on MnO_2 -reductase activity. The influence of temperature on the rate of glucose-linked, ferricyanide-dependent MnO_2 -reductase activity of uninduced whole cells was tested in fivefold diluted seawater, 0.1 M $NaCl$, and 0.05 M $MgCl_2 \cdot 6H_2O$ (Fig. 4). In all three cases the optimal temperature for the activity was in the range of 38 to 40 C. Although minimal and maximal temperatures were not determined in activity of uninduced whole cells, less than 20% of the maximal activity was detected at 15 and 50 C. It should be noted that at 25 C (Fig. 4), the incubation temperature most commonly used in previous experiments, the specific activity of whole cells was only about 45% of the maximal activity observed.

In extracts of uninduced cells, the optimal temperature for the glucose-linked, ferricyanide-dependent MnO_2 -reductase activity was 30 C (Fig. 5A). Approximately 65% of this activity was detected at 15 C, whereas 40% was detected at 15 C and 40% at 50 C. The ferrocyanide-linked MnO_2 -reductase activity of these extracts exhibited a temperature optimum near 40 C (Fig. 5B). The optimal temperature range of this activity was much broader than that of the glucose-linked, ferricyanide-dependent activities (Fig. 4 and 5A.)

The optimal temperature of the glucose-

TABLE 3. Effect of pre-exposure of whole cells of *Bacillus 29* to various cations in solutions on MnO_2 -reducing activity

Pre-exposure ^a	Reaction mixture ^b	Sp act ^c
Distilled water	Distilled water	0
Distilled water	0.05 M Mg^{2+}	249.0
0.059 M Mg^{2+}	0.05 M Mg^{2+}	377.0
0.059 M Mg^{2+}	0.008 M Mg^{2+}	399.0
0.05 M Mg^{2+} ; distilled water ^d	0.008 M Mg^{2+}	203.5
0.059 M Mg^{2+} ; distilled water ^e	0.008 M Mg^{2+}	171.0
Distilled water	0.05 M Ca^{2+}	274.0
0.059 M Ca^{2+}	0.05 M Ca^{2+}	302.0
0.059 M Ca^{2+}	0.008 M Ca^{2+}	301.0
Distilled water	0.014 M Na^+	59.0
Distilled water	0.1 M Na^+	198.0
0.1 M Na^+	0.014 M Na^+	41.0
0.1 M Na^+	0.1 M Na^+	225.0

^a Whole cells were washed twice in the solution listed.

^b Includes 0.0004 M K^+ from $K_3Fe(CN)_6$.

^c Glucose-linked, ferricyanide-dependent MnO_2 -reductase activity of uninduced whole cells. Specific activity expressed as nanomoles of Mn^{2+} released per hour per milligram of cell protein. Reaction mixtures were incubated at 25 C.

^d Cells washed with solutions containing Mg^{2+} were subsequently washed once with distilled water.

^e Cells washed twice with distilled water.

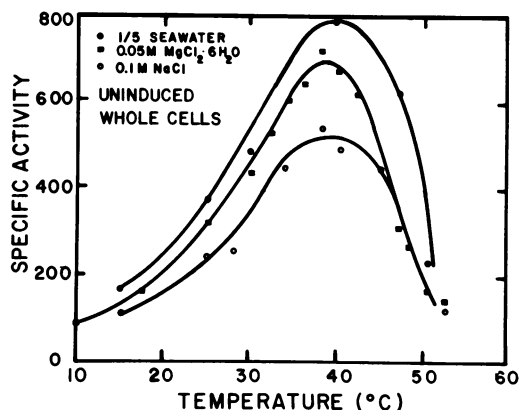


FIG. 4. Effect of incubation temperature on glucose-linked, ferricyanide-dependent MnO_2 -reductase activity in uninduced whole cells of *Bacillus 29*. Specific activity was expressed as nanomoles of Mn^{2+} released per hour per milligram of cell protein.

linked activity in induced whole cells was also found to be near 40 C, with very little activity detected at 15 or 50 C, (Fig. 6). In extracts this activity was greatest in mixtures incubated at temperatures near 25 C, with 70% of the maximal activity detected in mixtures incubated at 15 C and 60% at 10 C (Fig. 7). No activity was detected in induced cell extracts tested at 50 C.

DISCUSSION

The effects of seawater cations which were demonstrated in this work reflect the marine origin of *Bacillus* 29. The finding that the MnO_2 -reductase system was most stimulated by Na^+ and K^+ when these cations were present at lower concentrations than in natural seawater is not unlike the findings for enzymes derived from other marine bacteria (12-14).

Although not specifically investigated, it is appropriate to speculate about possible means

by which the individual cations influence the MnO_2 -reducing activity of *Bacillus* 29. By analogy with the effects of Na^+ and K^+ on other marine bacteria and their enzymes (12, 13), these cations most likely affect the MnO_2 -reductase activity both by regulating the activity of enzymes directly, and by affecting the permeability of the cell membrane to compounds essential for activity. In support of this analogy are the results of experiments showing that the Na^+ concentration maxima were different for cell extracts (Fig. 2, about 0.1 M) and whole cells (Fig. 1, about 0.25 M). In extracts, Na^+ presumably acted by interacting with enzymes of the system which were exposed to it. In whole cells, Na^+ could also act in this way by affecting either intracellular enzymes on the cell surface such as the terminal MnO_2 -reductase, which must be exposed to the extracellular environment to react with MnO_2 . Alternatively, Na^+ might affect the permeability of the cell membrane to glucose or ferricyanide, thus indirectly regulating the activity. The fact that both the glucose- and ferricyanide-linked systems of cell extracts responded to various concentrations of Na^+ in qualitatively the same way (Fig. 2 and 3) suggests that unless all enzymes in the electron transport system are equally sensitive to Na^+ , it is the terminal (ferricyanide-linked) portion of the system, perhaps the terminal reductase itself, which determines the response of the entire (glucose-linked) system to Na^+ . Thus, it is possible that the Na^+ effect on the MnO_2 -reductase activity of whole cells is due to the combined responses of the terminal enzyme of the system and membrane permeability to the presence of this cation.

Since K^+ resembled Na^+ in its effect on the MnO_2 -reductase system (i.e., Na^+ and K^+ concentration maxima corresponded to those in two- to fivefold diluted seawater [Fig. 1-3]), it seems likely that K^+ may also affect both the enzymes of the system and membrane permeability. MacLeod (12, 13) has previously shown that K^+ affects permeability to organic substrates in some marine bacteria.

Results showing that Ca^{2+} and Mg^{2+} elicited a biphasic stimulation of whole-cell activity (Fig. 1), as well as stimulating the activity of extracts (Fig. 2 and 3), suggest at least two possible roles for these cations. One role may be activation of the terminal reductase or other enzymes in the cell membrane which affect glucose transport. Such enzymes, specifically Mg^{2+} -activated adenosine triphosphatase which may function in active transport, have

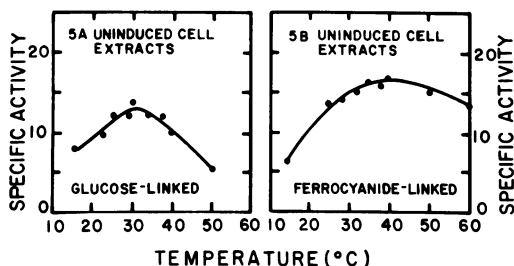


FIG. 5. Effect of incubation temperature on glucose-linked, ferricyanide-dependent and ferrocyanide-linked MnO_2 -reductase activities of extracts of uninduced cells of *Bacillus* 29. (A) glucose-linked, ferricyanide-dependent activity; (B) ferrocyanide-linked activity. Specific activity was expressed as nanomoles of Mn^{2+} released per hour per milligram of protein.

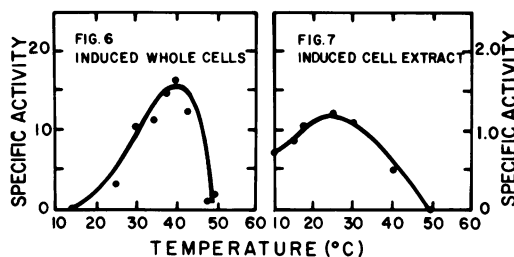


FIG. 6. Effect of incubation temperature on glucose-linked MnO_2 -reductase activity of uninduced whole cells of *Bacillus* 29. Specific activity was expressed as nanomoles of Mn^{2+} released per hour per milligram of cell protein.

FIG. 7. Effect of incubation temperature on glucose-linked MnO_2 -reductase activity of extracts of induced cells of *Bacillus* 29. Specific activity was expressed as nanomoles of Mn^{2+} released per hour per milligram of protein.

been found in the isolated cell envelopes of two marine pseudomonads and a *Cytophaga* (13). In addition, Mg^{2+} and Ca^{2+} may function to enhance binding of whole cells and/or the terminal MnO_2 -reductase to the mineral surface by the formation of ionic bridges between negative charges in the cell-wall matrix, or on the enzyme particles, (e.g., carboxyl and phosphate groups) and like charges on the surface of the MnO_2 particles. Such enhanced binding would bring the terminal reductase into closer proximity to its solid substrate, thus elevating the activity.

The binding-enhancement hypothesis is supported indirectly by pre-exposure experiments which indicated that Mg^{2+} and Ca^{2+} were much more tightly bound to the cells than Na^+ . These results parallel those of Cutinelli and Galdiero (1) who showed that the cell walls of *Staphylococcus aureus* behaved like weak cation-exchange resins, binding both monovalent and divalent cations by ionic bonds. Further, studies by Galdiero et al. (10) showed that the divalent cations were strongly bound by the cell walls, presumably due to electrostatic interactions with negative charges in the wall matrix. The monovalent cations were less strongly bound, appearing to form a mobile monolayer around the cell wall surface.

Thus, Na^+ , K^+ , Ca^{2+} , and Mg^{2+} may exert multiple effects on the MnO_2 -reducing activity of whole cells by influencing membrane permeability, active transport, and binding of enzymes or the cells to MnO_2 particles, as well as by directly regulating the activity of enzymes of the MnO_2 -reductase system itself.

Experiments testing the influence of various temperatures on the rate of MnO_2 -reductase activity (Fig. 4-7) indicated that the temperature optimum of the MnO_2 -reducing system has changed over the past five years. Previous experiments with induced and uninduced whole cells showed that the optimal temperatures of their respective MnO_2 -reductase activities were 25 and 30 C (unpublished data). Trimble and Ehrlich (16) found that the induced system in cell extracts was most active at 18 C. The present optimum for both induced and uninduced whole cells is 40 C (Fig. 5 and 6), and for induced cell extracts is 25 C (Fig. 7). These findings support earlier conclusions (7; Trimble, Ph.D. thesis, Rensselaer Polytechnic Institute, Troy, N.Y. 1969) that the temperature characteristics of *Bacillus* 29 have changed since its isolation more than 10 years ago. The changes may be attributable to the selective effects of cultivation of populations of this

organism at 25 C, which is higher than the temperature of its original marine habitat (5 to 10 C). Presumably, strains have developed from the original culture under the selective pressure of higher growth temperature which have MnO_2 -reducing enzymes with higher temperature optima than the parent culture.

The current experiments also indicate variation in the relative heat stabilities of the terminal and inducible portions of the MnO_2 -reductase system. The terminal (ferrocyanide-linked) portion of the system was less inhibited by temperatures of 50 and 60 C than the entire glucose-linked, ferricyanide-dependent system in cell extracts (Fig. 5A and B). This suggests that the terminal enzyme component is relatively more heat stable than other enzymes of the system. Heat-inactivation experiments, showing that the ferrocyanide-linked activity in soluble enzyme fraction of uninduced *Bacillus* 29 retained 60% of its original activity after 20 min in boiling water, whereas the glucose-linked activity of uninduced whole cells was completely inactive after 5 min of boiling (Ghiorse, Ph.D. thesis, Rensselaer Polytechnic Institute, Troy, N.Y. 1972), are consistent with the foregoing conclusion. The findings that enzymes in induced whole cells and extracts were inactive at 50 C (Fig. 6 and 7), whereas those in uninduced cells and extracts still had measurable residual activity at that temperature (Fig. 4 and 5), indicate that the inducible portion of the system is more heat-sensitive than other parts of the system. This heat sensitivity could explain why induced cell extracts had a lower temperature optimum than uninduced extracts.

The temperature characteristics of the MnO_2 -reductase system of *Bacillus* 29 are unusual. MnO_2 -reducing isolates from the Pacific Ocean studied in this laboratory exhibit stable temperature optima more in keeping with their original habitat (Ehrlich, unpublished data).

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