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Iron Respiration-Driven Proton Translocation in Aerobic Bacteria

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Washed cell suspensions of *Aquaspirillum magnetotacticum* MS-1, *A. itersonii* E12639, *Bacillus subtilis* 6633, and *Escherichia coli* CSH27 translocated protons in response to the added oxidant O_2 or NO_3^-, with triphenylmethylphosphonium bromide as the permeant ion. Iron respiration-driven proton translocation was observed in *A. magnetotacticum* MS-1, *B. subtilis*, and *E. coli* but not in a nonmagnetic strain of *A. magnetotacticum* (strain NM-1A) or with *A. itersonii*. Proton translocation to Fe^{3+} was totally inhibited by 500 μM NaN_3 or 0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone.

Bacterial respirations with O_2, NO_3^-, SO_4^{2-}, manganic ion, and other inorganic compounds as terminal oxidants have been subjects of numerous investigations. In contrast, physiological studies addressing dissimilatory Fe^{3+} reduction by microbes are almost nonexistent. Ferricyanide reduction with NADH by membrane vesicles of *Bacillus subtilis* (1) or with formate by *Escherichia coli* membrane vesicles (4) was linked to a functioning electron transport chain. It was suggested that there were two sites of ferricyanide reduction, one of which was the nitrate reductase. Under anaerobic conditions, ferricyanide reduction generated sufficient proton motive force to drive active transport of amino acids. Using membranes from cells of *Aquaspirillum itersonii*, Dailey and Lascelles (7) obtained evidence for Fe^{3+} reduction with NADH or succinate as the reductant. From studies with respiratory inhibitors, they concluded that the terminal Fe^{3+} reductase accepted electrons from donors at one or more sites before cytochrome *c* in the electron transport chain. It is extremely interesting that aeration abolished the Fe^{3+} reductase activity but not in the presence of antimycin A, suggesting that inhibition was due to "diversion of reductant into the terminal oxidase system" (7).

Lascelles and Burke (11) examined Fe^{3+} reduction by membranes of *Staphylococcus aureus* and, from results of studies with respiratory inhibitors, concluded that Fe^{3+} interacted with a reduced component of the electron transport chain that preceded cytochrome *b*.

Bacterial substrate oxidation is accompanied by extrusion of protons to the cell exterior (12). With a substrate in excess, protons are translocated across the cytoplasmic membrane in direct proportion to the quantity of an available suitable terminal electron acceptor. Proton translocation measurements (12) have been widely used to assess bacterial respiration with diverse oxidants. In this paper, we present evidence obtained with this technique for iron respiration in several species of aerobic bacteria.

Bacteria and culture conditions. *A. magnetotacticum* strains were cultured microaerobically at 28°C in static bottles containing MSGM medium, which contains 1.8 mM $NaNO_3$, 6 mM succinate, and 20 μM ferric quinate as the nitrogen, carbon, and Fe^{3+} sources, respectively (2). The nonmagnetic strain used in this study was a subclone of strain NM-1A. Nonmagnetic strain NM-1A was obtained from strain MS-1 by plating 0.1 ml (10^7) of cells on MSGM medium containing 0.005% sodium metabisulfite (1.0% agar). The plates were incubated in air for 14 days at 30°C. Two white, raised, circular colonies which appeared on one plate consisted of spirilla similar in size and morphology to cells of *A. magnetotacticum*. As expected of cells grown at high O_2 concentrations (2, 3), they lacked magnetosomes and, although motile, were not magnetotactic. Unlike cells of the parent colonies, cells from each of these colonies, when inoculated into liquid culture medium and grown microaerobically, failed to synthesize magnetosomes after more than 20 passages. Cells of strain NM-1A have been

TABLE 1. Proton translocation in the strains tested

Strain	Mean nmol of H^+ per g atom of oxidant measured with[a]:		
	O_2	NO_3^-	Fe^{3+}
A. magnetotacticum			
MS-1	6.0 (2.2–11.7) [15]	4.5 (0.8–9.6) [10]	2.6 (0.2–7.8) [14]
NM-1A	7.7 (7.0–8.2) [3]	5.1 (4.6–5.9) [3]	ND [4]
A. itersonii E12639	4.8 (1.3–9.7) [5]	7.7 (0.0–15.7) [4]	ND [5]
B. subtilis 6633	4.5 (1.6–9.1) [8]	2.8 (0.2–5.8) [7]	8.0 (0.5–24.8) [7]
E. coli CSH27	1.9 (1.1–2.6) [3]	5.7 (3.8–8.4) [3]	11.7 (8.6–15.4) [3]

[a] Numbers in parentheses indicate ranges; numbers in brackets indicate the numbers of cell preparations tested. Each cell preparation was tested repeatedly a minimum of four times with the oxidant shown. ND, Proton translocation not detected.

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shown to have guanine-plus-cytosine contents (64 to 65 mol%) and outer membrane protein profiles similar to those of strain MS-1. *A. itersonii* E12639 (University of New Hampshire culture collection) was cultured with swirling (100 rpm) at 30°C in Fernbach flasks containing MSGM medium plus 0.1% yeast extract. *B. subtilis* 6633 and *E. coli* CSH27 (University of New Hampshire culture collection) were cultured with swirling (100 rpm) at 37°C in Fernbach flasks containing tryptic soy medium (Difco Laboratories, Detroit, Mich.).

Cell preparations. Cells were harvested at the mid to late exponential phase of growth by using a Pellicon filtration system (Millipore Corp, Bedford, Mass.) and were concentrated by centrifugation. They were washed twice in 150 mM KCl (pH 7.1) at 4°C and resuspended in 10 ml of the same to a density of more than 2×10^{10} cells ml^{-1}.

Oxidant pulse studies. The apparatus, reagents, and experimental techniques used for oxidant pulse studies were those of Kristjansson et al. (10) and Castignetti and Hollocher (5). The reaction vessel was a 5-ml vial containing a small magnetic stir bar and fitted with a rubber closure. A combination pH electrode (Value Mark model H-445; Markson Science) and needles for gas inlet and outlet were inserted through the closure. A Beckman Altex model 71 pH meter was used with a recorder set to 0.1 pH unit, full scale. To a 2-ml cell suspension (in KCl) in the vial were added triphenylmethylphosphonium bromide ($TPMP^+$) at 0.6 mM and 7.2 U of carbonic anhydrase. The contents were then flushed with O_2-free N_2 and continuously stirred for 20 min to render them anaerobic. Subsequently, the pH of the suspension was adjusted to ca. 7.1 with an anaerobic solution of 0.01 N NaOH.

Chemicals were prepared in gas-tight serum vials as dissolved gasses or solids in 150 mM KCl and made anaerobic by first heating the contents to 45°C and then cooling them with repeated evacuation of the headspace, followed by replacement with 1 atm (101.29 kPa) of O_2-free N_2 immediately prior to use. For tests with O_2, a known quantity of pure O_2 was equilibrated in 4 ml of 150 mM KCl in a 9-ml serum vial prepared as described above. Dissolved O_2 was calculated by assuming 1.16 mM O_2 atm^{-1} (6). Nitrate was prepared as 2 mM $NaNO_3$ in 150 mM KCl. Iron was prepared as ferric quinate (2 mM) or ferric chloride (2 or 5 mM) in 150 mM KCl. The pH of chemicals tested as potential oxidants was adjusted to 7.10.

Iron reduction and nitrate reductase. Iron reduction by intact cells was determined with Ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine; Sigma Chemical Co., St. Louis, Mo.] (13). Nitrate reductase was assayed in cell-free extracts by coupling to NADH oxidation (9).

Cells contained sufficient endogenous substrate for respiration, as determined by O_2 consumption rates measured with a polarographic electrode (YSI model 53 oxygen monitor). They generated proton pulses repeatedly for up to several hours with each addition of suitable oxidant. Pulse amplitudes were quantified by using the graphic methods of Scholes and Mitchell (12). The magnitude of the measured proton pulse was correlated with the molar concentration of extruded protons by injecting a known quantity of anaerobic 0.01 N HCl. The effects of oxidants and inhibitors were tested by adding appropriate quantities as anaerobic solutions directly to the reaction vessel.

Washed cell suspensions of *B. subtilis* 6633, *E. coli* CSH27, and *A. magnetotacticum* MS-1 but not nonmagnetic *A. magnetotacticum* NM-1A or *A. itersonii* E12639 (Table 1) demonstrated proton translocation in response to added

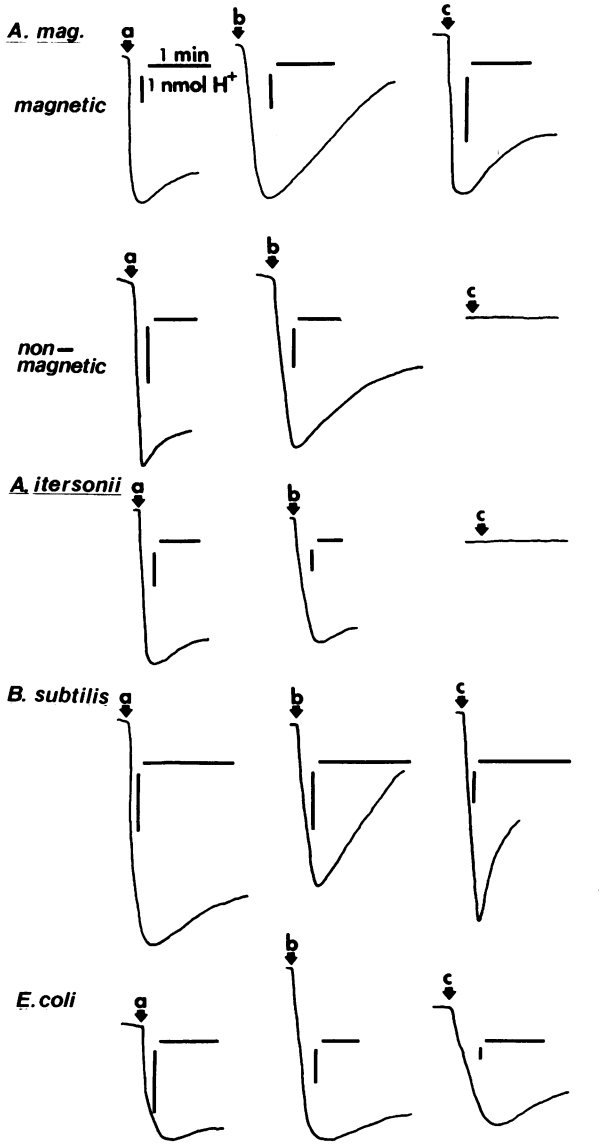

FIG. 1. Proton pulse traces obtained with cells of *A. magnetotacticum* (*A. mag.*) (magnetic and nonmagnetic strains), *A. itersonii*, *B. subtilis*, and *E. coli* in the presence of 0.6 mM $TPMP^+$ and 7.2 U of carbonic anhydrase. Arrows indicate the addition of the following oxidants: a, 1 ng-atom of O_2; b, 1 ng-atom of NO_3^-; and c, 1 ng-atom of Fe^{3+}. Horizontal-scale bars equal 1 min. Vertical-scale bars equal 1 nmol of H^+ translocated to the cell exterior.

Fe^{3+} with 0.6 mM $TPMP^+$ as the permeant ion. Washed cell suspensions of strain MS-1 similarly prepared actively reduced added Fe^{3+} (2.5 ng of Fe^{3+} reduced μg of cell $protein^{-1}$ min^{-1}). *A. itersonii* cell suspensions equilibrated with 0.3, 0.6, or 1.2 mM $TPMP^+$ or with 0.6 mM valinomycin generated proton pulses with O_2 and NO_3^- but not with Fe^{3+}. Cells of all species tested translocated protons in response to added O_2 and NO_3^-, as expected of these dissimilatory nitrate reducers. These results suggested that many cells in cultures of these organisms became sufficiently anaerobic by the end of growth to be anoxia derepressed for nitrate reductase. The specific activities of nitrate reductase in cell-free extracts of *A. itersonii*, *B. subtilis*, and *E. coli* were 8×10^{-2}, 2×10^{-1}, and 6×10^{-1} μmol mg of $protein^{-1}$ min^{-1}, respectively. Representative proton pulse curves for

each species supplied with the oxidants examined in this study are shown in Fig. 1. The proton gradients showed decay periods varying in magnitude. We attribute this to the use of $TPMP^+$, which causes membrane leakiness to H^+ in a species-dependent manner (5). *E. coli* appeared to have a membrane less permeable to H^+ than did the other species when this protonophore was used. Proton translocation by each organism was abolished by the addition of 0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone, regardless of the acceptor. In some experiments (particularly with Fe^{3+}), the magnitude of the pulse was much greater than the mean value. Hence, the range and the mean for each oxidant are presented. We did not observe proton pulses when any of the bacteria were tested with fumarate, dimethyl sulfoxide, or manganese dioxide.

Proton translocation by *A. magnetotacticum* MS-1 with either NO_3^- or Fe^{3+} was completely inhibited by 250 μM NaN_3, while O_2-driven proton translocation was decreased by 40%. This concentration of NaN_3 decreased cell oxygen consumption by 70%. Proton translocation by *E. coli* and *B. subtilis* with Fe^{3+} was completely inhibited by 500 and 250 μM NaN_3, respectively. These results are consistent with iron reduction at a terminal site in the electron transport chain.

Our results demonstrate that intact cells of three bacterial species cultured aerobically translocate protons in response to added Fe^{3+} and, presumably, are therefore capable of generating a proton motive force via the dissimilatory reduction of Fe^{3+}. Differences between magnetic and nonmagnetic cells of *A. magnetotacticum* could relate to the ability of magnetic cells to reduce Fe^{3+} in a dissimilatory manner, perhaps as a result either of not being able to transport Fe^{3+} to a site of reduction or of an inability to produce a specific iron reductase. Bacterial magnetite formation involves Fe^{3+} reduction (8). Microaerobic aquatic habitats from which magnetic bacteria have been collected are typically low in O_2 and NO_3^- but relatively high (20 μM or more) in Fe^{3+}. The ability of this organism to respire with Fe^{3+} could provide a competitive advantage in these habitats.

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