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Regulation of Dissimilatory Fe(III) Reduction Activity in *Shewanella putrefaciens*

ROBERT G. ARNOLD,[1]* MICHAEL R. HOFFMANN,[2] THOMAS J. DiCHRISTINA,[3] AND FLYNN W. PICARDAL[1]

Department of Civil Engineering and Engineering Mechanics, University of Arizona, Tucson, Arizona 85721[1]; Department of Environmental Sciences and Engineering, California Institute of Technology, Pasadena, California 91125[2]; and Woods Hole Oceanographic Institute, Redfield Laboratory, Woods Hole, Massachusetts 02540[3]

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Under anaerobic conditions, *Shewanella putrefaciens* is capable of respiratory-chain-linked, high-rate dissimilatory iron reduction via both a constitutive and inducible Fe(III)-reducing system. In the presence of low levels of dissolved oxygen, however, iron reduction by this microorganism is extremely slow. Fe(II)-trapping experiments in which Fe(III) and O_2 were presented simultaneously to batch cultures of *S. putrefaciens* indicated that autoxidation of Fe(II) was not responsible for the absence of Fe(III) reduction. Inhibition of cytochrome oxidase with CN^- resulted in a high rate of Fe(III) reduction in the presence of dissolved O_2, which suggested that respiratory control mechanisms did not involve inhibition of Fe(III) reductase activities or Fe(III) transport by molecular oxygen. Decreasing the intracellular ATP concentrations by using an uncoupler, 2,4-dinitrophenol, did not increase Fe(III) reduction, indicating that the reduction rate was not controlled by the energy status of the cell. Control of electron transport at branch points could account for the observed pattern of respiration in the presence of the competing electron acceptors Fe(III) and O_2.

The importance of dissimilatory iron reduction as a determinant of the organic carbon mineralization rate and iron bioavailability has been studied within coastal sediments (25), anoxic lake sediments (10, 11, 28), and estuarine waters (13–15). There has been considerable speculation that iron assimilation can limit biological activity in a variety of marine environments (1, 2, 6, 8, 17, 22). Dissimilatory iron reduction may also have important implications for soil weathering and consequences for groundwater quality (4). In addition, iron-reducing microorganisms have been implicated in accelerated pipeline corrosion (19, 20; C. O. Obuekwe, Ph.D. dissertation, University of Alberta, Edmonton, Alberta, Canada, 1980).

Direct biological reduction of Fe(III) by bacteria has been demonstrated in only three known cases. Lovley and Phillips (16) isolated a gram-negative rod, designated GS-15, from sediments of the Potomac River. GS-15 was capable of coupling growth to electron transport from a variety of electron donors to Fe(III). Myers and Nealson (18) isolated a gram-negative, facultative anaerobe, *Alteromonas putrefaciens* MR-1, which grew anaerobically with Fe(III) as the terminal electron acceptor. Similarly, Arnold et al. (4) found that an oil pipeline isolate, subsequently designated *Shewanella putrefaciens* sp. strain 200 (23), catalyzed the reductive dissolution of goethite and hematite only when direct contact with the mineral surface was possible.

Rates of dissimilatory iron reduction by *S. putrefaciens* sp. strain 200 normalized for cell density are among the highest reported. Arnold et al. (5) found that the kinetics of dissimilatory iron reduction by *S. putrefaciens* depend upon the equilibrium concentrations of individual Fe(III) species other than free ferric iron. At high equimolar concentrations of total Fe(III) and the iron chelator nitrilotriacetic acid (NTA) (1.86×10^{-3} M), electron transfer to Fe(III) was limited by the same factors as aerobic respiration, presumably at the dehydrogenase level (9).

In *S. putrefaciens*, the dissimilatory iron reduction capacity increased nearly 10-fold upon induction via growth under O_2-limited conditions: less than 1% of saturating levels of dissolved oxygen, or about 2.5 μM (5). Electron transfer in the presence of specific respiratory inhibitors indicated that the species harbors both inducible and constitutive Fe(III)-reductase activities. Iron reduction via the constitutive system was blocked by CN^-, dicyclohexylcarbodiimide (DCCD), 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), dicumarol, and quinacrine. The inducible system was uninhibited by CN^-, N_3^-, HQNO, and DCCD. The inability of DCCD to interrupt dissimilatory iron reduction among cells grown at low oxygen tension suggests that electron transport via the high-rate, inducible iron reductase system is not coupled to oxidative phosphorylation (3). Thermodynamics indicate that dissimilatory iron reduction occurs via an abbreviated electron transport chain (27). Inhibitor studies suggest that the constitutive Fe(III)-reductase activity is located farther (downstream) along the electron transport chain than is its inducible counterpart.

Fe(III) reduction, which would be energetically wasteful in the presence of more thermodynamically favorable electron acceptors such as O_2, has not been observed under aerobic conditions. Any of the following mechanisms could account for the apparent lack of Fe(III) reduction by *S. putrefaciens* in the simultaneous presence of Fe(III) and O_2. (i) Biochemically reduced ferrous iron could be rapidly autoxidized to its original Fe(III) form in the presence of dissolved oxygen. In fact, autoxidation of Fe(II) is accelerated by iron chelators such as NTA (12). (ii) Fe(III)-reductase activities could be directly inhibited by dissolved oxygen. (iii) There may also be interference with transmembrane transport of Fe(III) by dissolved oxygen, although it has never been shown that such transport is required for dissimilatory iron reduction. (iv) Control may arise from the energy status of the cell; low energy levels could direct electron flow to alternative electron acceptors by an unknown mechanism. (v) Finally, the kinetics of competing reactions at branch points in the electron transport chain could direct reducing

* Corresponding author.

equivalents to O_2 in the presence of Fe(III). In this paper we describe experiments designed to determine which of these hypothetical mechanisms is responsible for the control of Fe(III) reduction by *S. putrefaciens* sp. strain 200 in the presence of molecular oxygen.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. putrefaciens* sp. strain 200 is a gram-negative, motile rod. The strain used in these experiments was isolated by Obuekwe from a Canadian oil pipeline (Obuekwe, Ph.D. dissertation). Initial confusion about the species identity (previously *Pseudomonas* sp. strain 200, *P. ferrireductans*, and *Alteromonas putrefaciens* sp. strain 200) has been resolved in favor of its present identification (23, 24).

In the experiments measuring Fe(III) reduction in the presence of oxygen, *S. putrefaciens* was grown in 1.5-liter batch cultures (Biostat M fermentor; Braun Instrument Co.) at 30°C to a target optical density of $A_{600} = 0.15$. Westlake medium (pH 7.0) was prepared by the method of Obuekwe et al. (19). Growth was initiated by the addition of 0.5 ml of a dense culture grown overnight in identical medium. Cultures were maintained under high (>190 μM) or low (<2.5 μM) oxygen tension during growth via manipulation of air flow and culture agitation rate to favor the constitutive or inducible ferrireductase activities, respectively. At the target optical density, chloramphenicol (Sigma Chemical Co.) was added to a final concentration of 2.3×10^{-4} M. NTA and $FeCl_3$ were then each added to a final concentration of 1.86×10^{-3} M prior to the interruption of air flow to the reactor and initiation of continuous dissolved oxygen (Ingold O_2 probe) and periodic total ferrous iron measurements. CN^- was added incrementally to concentrations previously established as inhibitory to aerobic respiration in *S. putrefaciens* (3).

The experiments to determine whether control of Fe(III) reduction was based on cellular energy status involved growth of *S. putrefaciens* in 2-liter batch cultures (VirTis Omni-Culture fermentor). The growth medium, growth conditions, and inoculation procedures were identical to those used above. Manipulation of aeration and agitation rates were again used to maintain either high (>190 μM) or low (<2.5 μM) oxygen tension during growth, thus favoring the presence of either the constitutive or inducible Fe(III) reductase. Chloramphenicol was not used in this portion of the experiment since an inhibitor of protein synthesis might have produced artificially high intracellular ATP concentrations. A 50-ml sample of the culture was removed and placed in a flask in a water bath at 30°C with agitation. An uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP), was then added to a final concentration of 10^{-4} M. Preliminary experiments showed that 10^{-4} M DNP reduced the intracellular ATP concentration while allowing growth and respiration to continue. NTA and $FeCl_3$ were each added to a final concentration of 1.86×10^{-3} M, and samples were taken periodically for ATP and ferrous iron analyses. Oxygen uptake rates were simultaneously measured by using a Yellow Springs Instrument Co. model 5300 biological oxygen monitor with stir-bath assembly.

Fe(II) measurement. To eliminate autoxidation of Fe(II), we added 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine; Sigma) to respiring cultures. The procedure was derived from that of Carter (7). The stability constant for the 1:3 [Fe(II)-ferrozine] bidentate complex is too high for measurement by conventional means (26), and the complex forms on a time scale faster than autoxidation. To halt iron reduction at the point of sample withdrawal, samples were quick-frozen in dry ice. Prior to spectrophotometric measurement of the ferrozine-Fe(II) complex ($\epsilon_{562} = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$), samples were quickly thawed and centrifuged at 4°C for 5 min at 3,000 × *g* in a Sorvall model RC-3B centrifuge or an Eppendorf model 5415 microcentrifuge. Absorbance measurements were made by using a Beckman model DU7 spectrophotometer or Shimadzu UV-160A spectrophotometer.

ATP measurement. ATP levels were determined by a modification of the method described by Patterson et al. (21). ATP was measured by adding 1.0 ml of the culture to 25 ml of hot (90°C) buffer solution consisting of 0.025 M Tris buffer (J. T. Baker Chemical Co.) and 0.002 M disodium EDTA (Aldrich Chemical Co., Inc.). After 10 min, the mixture was cooled to room temperature. ATP concentrations in the mixture were determined by using firefly extract (stock FLE-50; Sigma) and a Turner Designs model TD-20e luminometer.

RESULTS AND DISCUSSION

Controls. Control experiments indicated that ferrozine neither reduced Fe(III) abiotically under experimental conditions nor inhibited aerobic respiration in cultures of *S. putrefaciens*. The anaerobic, abiotic rate of Fe(III) reduction was extremely low: 7.0×10^{-6} M h^{-1}. When the abiotic control experiment was repeated in the presence of saturating levels of dissolved oxygen, no Fe(III) reduction was observed. Under highly aerobic conditions, the rate of O_2 utilization was unaffected by the addition of ferrozine (1.78×10^{-3} M). Upon the addition of 2.3×10^{-4} M chloramphenicol, bacterial growth ceased immediately, but oxygen demand was virtually unaffected during the 50 min required for completion of the experiments.

Ferrozine trap experiments. (i) O_2 and Fe(III) presented simultaneously. Experiments (Fig. 1) involving the cultures grown under conditions favoring the constitutive ferrireductase system (not O_2 limited during growth) indicated that Fe(III) reduction by *S. putrefaciens* is slow (1.2×10^{-5} M h^{-1}) in the presence of even low concentrations of dissolved oxygen, although nearly twice as fast as abiotic rates. Anticipated iron reduction rates were not achieved until residual oxygen was purged from both the medium and reactor headspace with high-purity N_2. Assuming that O_2 reduction involves a four-electron transfer, electron transport to O_2 was about 1 order of magnitude faster than electron transport to Fe(III) following O_2 exhaustion. The iron reduction rate following O_2 exhaustion (4.1×10^{-6} M min^{-1}) was similar to rates measured in previous Fe(III)-reducing experiments involving *S. putrefaciens* in which the production of Fe(II) was determined by phenanthroline-based assays (5).

The experimental procedure was repeated with cultures of *S. putrefaciens* grown under O_2-limited conditions to favor the presence of inducible ferrireductase activity. Dissimilatory iron reduction proceeded only after virtual exhaustion of dissolved oxygen (Fig. 2). The maximum rate of electron transfer to Fe(III) was approximately equal to the rate of electron transfer to O_2 (5.3×10^{-5} and 6.3×10^{-5} M e^- min^{-1}, respectively), again assuming a four-electron transfer for O_2 reduction. This suggested that dissimilatory iron reduction by induced cultures was subject to the same kinetic limitations as electron transfer to oxygen.

(ii) CN^- inhibition of aerobic respiration. CN^- was added

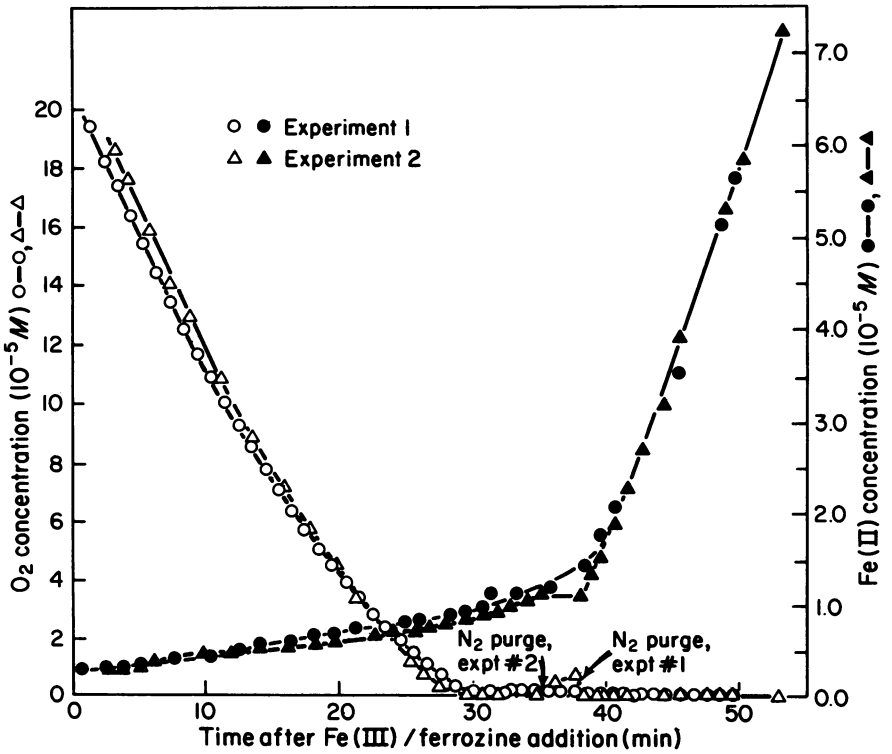

FIG. 1. Duplicate ferrozine trap experiments in which dissolved oxygen utilization and iron reduction were measured simultaneously. Cells were grown to A_{600} = 0.15 under highly aerobic conditions prior to the introduction of high-level Fe(III), NTA, and ferrozine.

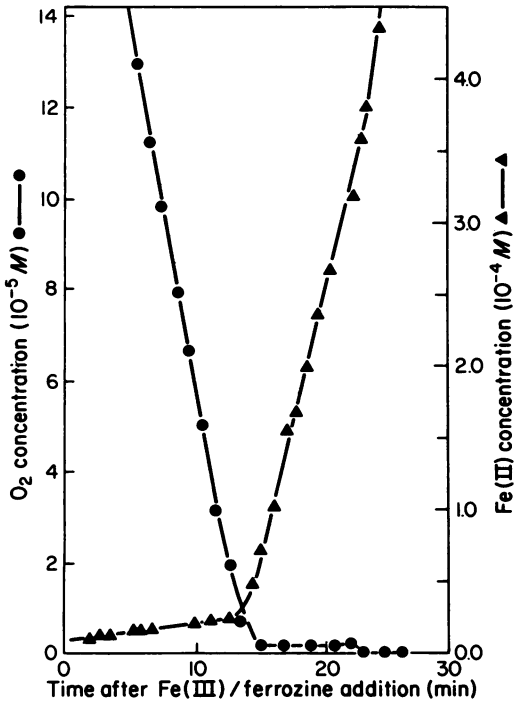

FIG. 2. Simultaneous O_2 utilization and Fe(III) reduction in a pure, batch culture of *S. putrefaciens* (A_{600} = 0.15) grown under low-O_2 (inducing) conditions. Concentrations: Total Fe, 1.86×10^{-3} M; NTA, 1.86×10^{-3} M; ferrozine, 2.79×10^{-3} M.

incrementally to uninduced cultures of *S. putrefaciens* in the presence of both Fe(III) and dissolved oxygen (Fig. 3). At the target optical density (A_{600} = 0.15), the uninhibited O_2 utilization rate was 1.8×10^{-5} M min^{-1}. As expected, the O_2 utilization rate decreased incrementally with sequential additions of CN^- (Table 1). However, the iron reduction rate remained reasonably steady, between 2.17×10^{-6} and 2.50×10^{-6} M min^{-1}, after the first cyanide addition (Fig. 3). Iron reduction activity increased only slightly (to 3.0×10^{-6} M min^{-1}) in response to the purge of residual O_2. Iron reduction rates measured in the presence of 10^{-3} M CN^- were slightly lower than the rates observed in uninduced cultures under anaerobic conditions (4.1×10^{-6} M min^{-1}), suggesting that the constitutive ferrireductase activity is partially inhibited by CN^- at that level.

The results of similar CN^- inhibition experiments conducted with cells grown under O_2-limited (inducing) conditions are summarized in Fig. 4 and Table 1. CN^- was previously shown to be ineffective as an inhibitor of inducible ferrireductase activity in *S. putrefaciens* (3). In the absence of CN^-, electron transport was almost exclusively to O_2 when both molecular oxygen and complexed Fe(III) were present as potential electron acceptors. The onset of high-rate Fe(III) reduction coincided with partial CN^- inhibition of aerobic respiration. Neither additions of CN^- beyond 3×10^{-5} M (the maximum concentration used in these experiments) nor stripping of residual O_2 from solution contributed substantially to the observed rate of dissimilatory iron reduction. Maximal iron reduction activity was observed when the rate of oxygen utilization was only 57% inhibited. Greater inhibition of the cytochrome oxidase did not increase the rate of Fe(III) reduction. The maximum ferrireductase activity (5.0×10^{-6} M e^- min^{-1}) was not as

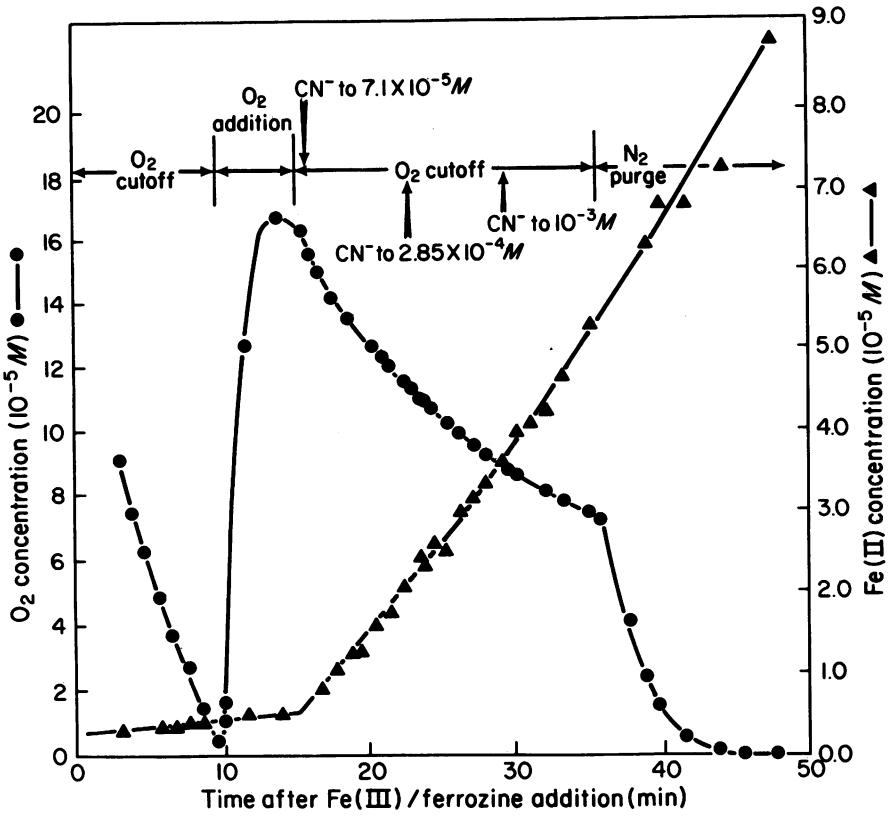

FIG. 3. Simultaneous O_2 utilization and Fe(III) reduction as a function of CN^- and O_2 concentrations in an uninduced culture of *S. putrefaciens* (A_{600} = 0.15). Iron reduction was measured in the presence of dissolved oxygen with ferrozine as a ferrous iron trap. Concentrations are the same as in the legend to Fig. 2.

high as expected from either previous iron reduction experiments conducted at higher optical densities or the uninhibited rate of dissolved oxygen utilization. It is possible that induction of the second ferrireductase system is a function of mean cell age as well as the O_2 level during growth.

(iii) Lowered intracellular ATP concentrations. Addition of 10^{-4} M DNP to the culture grown under high oxygen tension (uninduced culture) resulted in a 53% decrease in ATP levels relative to the untreated control. Measured ATP concentrations were corrected by dividing by the culture optical density (λ = 600 nm) to compensate for differences in cell number. The reported decrease in ATP concentration represents the mean of nine samples over a 60-min period. The addition of DNP also resulted in a 66% decrease in oxygen uptake rate and a retardation of the specific growth rate.

Fe(III) reduction for the uninduced culture in the presence of O_2, with and without DNP, is shown in Fig. 5. Rates of ferric iron reduction were similar for both cultures until the point at which oxygen was depleted in the culture containing DNP. Fe(III) reduction increased dramatically at that point, suggesting that the presence of molecular oxygen rather than the intracellular ATP concentration controls the rate of iron reduction by the constitutive enzyme activity.

The experiment with the culture grown under low oxygen tension (i.e., the induced culture) produced similar results. DNP at 10^{-4} M caused a 30% reduction in ATP levels, a 31% reduction in oxygen utilization, and a retardation of the specific growth rate. Fe(III) reduction for the induced culture in the presence of O_2, with and without DNP, is shown in Fig. 6. Rates of Fe(III) reduction were again similar for

TABLE 1. Simultaneous measurements of O_2 utilization and Fe(III) reduction as a function of CN^- concentration in *S. putrefaciens*

Growth conditions[a]	Total [CN] (M)	O_2 utilization rate (M min^{-1})	% Inhibition of O_2 utilization	Fe(III) reduction rate (M min^{-1})
Uninduced (A_{600} = 0.15)	0	1.8×10^{-5}	0	
	7.14×10^{-5}	5.2×10^{-6}	71	2.17×10^{-6}
	2.85×10^{-4}	3.9×10^{-6}	78	2.33×10^{-6}
	1.0×10^{-3}	2.3×10^{-6}	87	2.50×10^{-6}
Induced (A_{600} = 0.05)	0	9.6×10^{-6}	0	ca. 0
	3×10^{-5}	4.1×10^{-6}	57	5.0×10^{-6}
	3×10^{-4}	2.7×10^{-6}	72	5.0×10^{-6}
	3×10^{-3}	2.2×10^{-6}	77	5.0×10^{-6}

[a] Uninduced and induced cultures were grown with dissolved oxygen concentrations in excess of 190 μM and below 2.5 μM, respectively. The optical densities of cultures used in rate determinations are shown.

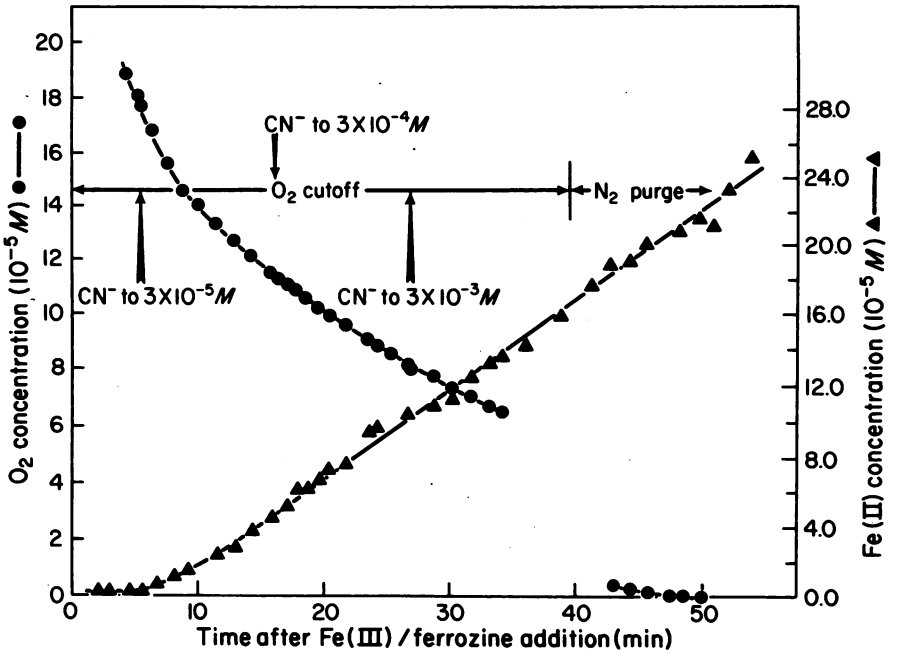

FIG. 4. Simultaneous O_2 utilization and Fe(III) reduction as a function of O_2 and CN^- concentrations in an induced culture of *S. putrefaciens* (A_{600} = 0.05). Iron reduction was measured in the presence of dissolved O_2 with ferrozine as a ferrous iron trap. Concentrations are the same as in the legend to Fig. 2.

both cultures until the point at which oxygen was depleted in the culture containing DNP. Control of Fe(III) reduction was provided not by the energy status of the cell (i.e., the ATP concentration), but by the dissolved oxygen concentration. It is also evident from these results that neither the constitutive nor the inducible ferrireductase activity was completely inhibited by DNP addition.

Implications for respiration control mechanisms. In the

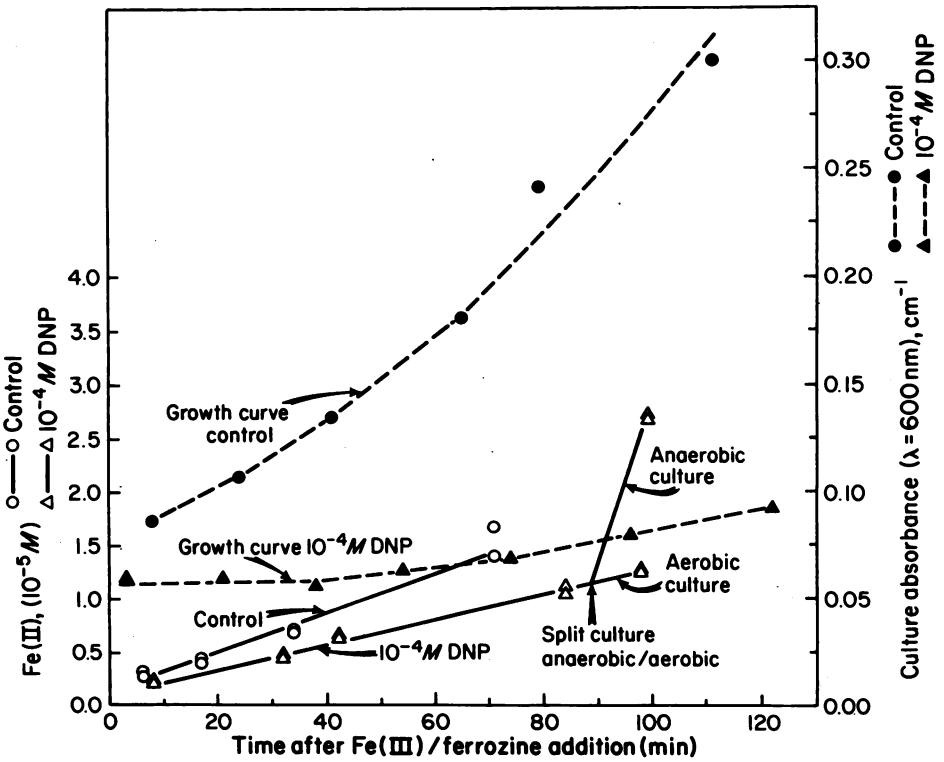

FIG. 5. Fe(III) reduction by uninduced cultures (grown under high-O_2 conditions) of *S. putrefaciens* with and without DNP (provided to lower intracellular ATP concentrations). Iron reduction was measured in the presence of dissolved O_2 with ferrozine as a ferrous iron trap. Concentrations are the same as in the legend to Fig. 2.

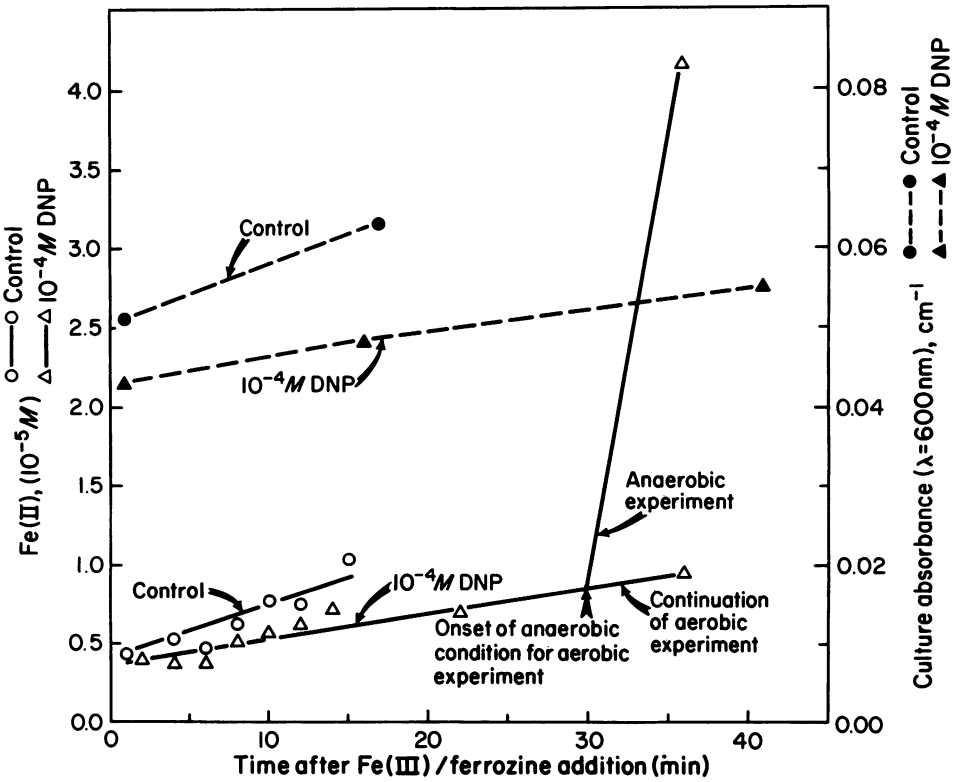

FIG. 6. Fe(III) reduction by induced cultures (grown under O_2-limited conditions) of *S. putrefaciens* with and without DNP (provided to lower intracellular ATP concentrations). Iron reduction was measured in the presence of dissolved O_2 with ferrozine as a ferrous iron trap. Concentrations are the same as in the legend to Fig. 2.

presence of O_2, Fe(III) is reduced at extremely low rates by either the induced or the uninduced iron reduction systems of *S. putrefaciens*. Ferrozine trap experiments indicate that autoxidation is not responsible for the apparent lack of ferrireductase activity under aerobic conditions. Simultaneous measurements of ATP levels and iron reduction during the experiments with DNP suggest that cellular energy status is not a major control factor. Cyanide inhibition experiments indicate that (i) neither the constitutive nor the inducible Fe(III)-reduction activity is directly inhibited by molecular oxygen and (ii) control mechanisms involving interference with transmembrane Fe(III) transport by O_2 are not supported by these data.

Although the regulatory mechanism in *S. putrefaciens* remains unknown, speculation is possible. The measured iron reduction rates are consistent with a mechanism which involves regulation of electron flow at respiratory-chain branch points separating pathways to the ferrireductase activities and cytochrome oxidase. The observed pattern of oxygen utilization and Fe(III) reduction would occur if electron transport from such a branch point were much faster in the direction of O_2 than of Fe(III). If E_{ox} and E_{red} represent the oxidized and reduced concentrations of an electron carrier at the branch point to O_2 and Fe(III), and K_{O_2} and $K_{Fe(III)}$ are rate constants specific to each reaction, then $K_{O_2} E_{red}$ is the maximum rate of electron transport to molecular oxygen and $K_{Fe(III)} E_{red}$ is the maximum rate of dissimilatory iron reduction. A control scheme based on branch point kinetics would require that K_{O_2} greatly exceed $K_{Fe(III)}$. Furthermore, iron reduction would be limited by electron transport to O_2 only if there were competition for reducing equivalents at the branch point. Consequently, the hypothetical scheme also requires that $E_{ox} \gg E_{red}$ or, equivalently, that O_2 utilization kinetics are limited ahead of the branch point to Fe(III).

Although this proposed regulatory mechanism is speculative, the data described in these experiments are consistent with such an explanation. Additional experiments are needed both to confirm postulated electron transport chain relationships and to identify carriers which serve as branch points to Fe(III).

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