JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1891–1896
0021-9193/92/061891-06$02.00/0

Vol. 174, No. 6

Effects of Nitrate and Nitrite on Dissimilatory Iron Reduction by *Shewanella putrefaciens* 200†

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Received 23 September 1991/Accepted 10 January 1992

The inhibitory effects of nitrate (NO_3^-) and nitrite (NO_2^-) on dissimilatory iron (Fe^{3+}) reduction were examined in a series of electron acceptor competition experiments using *Shewanella putrefaciens* 200 as a model iron-reducing microorganism. *S. putrefaciens* 200 was found to express low-rate nitrate reductase, nitrite reductase, and ferrireductase activity after growth under highly aerobic conditions and greatly elevated rates of each reductase activity after growth under microaerobic conditions. The effects of NO_3^- and NO_2^- on the Fe^{3+} reduction activity of both aerobically and microaerobically grown cells appeared to follow a consistent pattern; in the presence of Fe^{3+} and either NO_3^- or NO_2^-, dissimilatory Fe^{3+} and nitrogen oxide reduction occurred simultaneously. Nitrogen oxide reduction was not affected by the presence of Fe^{3+}, suggesting that *S. putrefaciens* 200 expressed a set of at least three physiologically distinct terminal reductases that served as electron donors to NO_3^-, NO_2^-, and Fe^{3+}. However, Fe^{3+} reduction was partially inhibited by the presence of either NO_3^- or NO_2^-. An in situ ferrozine assay was used to distinguish the biological and chemical components of the observed inhibitory effects. Rate data indicated that neither NO_3^- nor NO_2^- acted as a chemical oxidant of bacterially produced Fe^{2+}. In addition, the decrease in Fe^{3+} reduction activity observed in the presence of both NO_3^- and NO_2^- was identical to the decrease observed in the presence of NO_2^- alone. These results suggest that bacterially produced NO_2^- is responsible for inhibiting electron transport to Fe^{3+}.

The physiological role of nitrate reductase in dissimilatory iron reduction has been a matter of controversy since Ottow (13) first proposed that nitrate reductase could serve as an electron donor for both nitrate (NO_3^-) and ferric iron (Fe^{3+}). Several previous studies (12, 14, 20) demonstrated an apparent inhibitory effect of NO_3^- on the Fe^{3+} reduction activity of a variety of microorganisms grown under laboratory conditions. Ottow (13) suggested that nitrate reductase could transfer electrons to either NO_3^- or Fe^{3+} but would preferentially utilize NO_3^- in the presence of both oxidants. Thus, the lack of Fe^{3+} reduction activity was apparently due to competitive inhibition by NO_3^-. Subsequently, several investigators hypothesized that NO_3^- inhibition of Fe^{3+} reductase activity was primarily the result of secondary (i.e., postreduction) chemical reoxidation of bacterially produced ferrous iron (Fe^{2+}) by nitrite (NO_2^-) (6, 11).

Difficulty in separating the biological and chemical components of the Fe^{3+} reduction process is generally attributed to the high redox activity of iron salts at neutral pH (3, 19). For example, Fe^{3+} is reduced chemically by a variety of organic and inorganic compounds (e.g., ascorbate, phenols, and sulfides), whereas Fe^{2+} is oxidized chemically by a number of inorganic compounds, most notably molecular oxygen (O_2), manganese oxide [MnO_2 (s)], and NO_2^- (4, 9, 19).

The present study was undertaken to clarify the physiological relationship between the dissimilatory reduction of NO_3^-, NO_2^-, and Fe^{3+} and to determine whether the inhibitory effect of NO_3^- on Fe^{3+} reduction was due to competitive inhibition by NO_3^- or to chemical reoxidation of bacterially produced Fe^{2+} by NO_2^-. To meet these objectives, a series of electron acceptor competition experiments were conducted in which Fe^{3+} and either NO_3^- or NO_2^- were provided simultaneously to a model microorganism and the respective electron acceptor reduction activities were determined. An in situ Fe^{2+} production assay was used to minimize the effects of nitrogen oxide-catalyzed Fe^{2+} oxidation and to allow comparison of the biological and chemical components of the Fe^{3+} reduction process. *S. putrefaciens* 200 was chosen as a model microorganism for this study; it was selected largely because of its ability to reduce a wide range of terminal electron acceptors, including O_2, Fe^{3+}, NO_3^-, and NO_2^- (11, 15, 16). Results from earlier competition experiments with *S. putrefaciens* 200 demonstrated that the inhibitory effects of O_2 on Fe^{3+} reduction activity were due to preferential channeling of electrons to O_2 (2), whereas the inhibitory effects of NO_3^- on Fe^{3+} reduction activity were due to chemical reoxidation of bacterially produced Fe^{2+} by the product of NO_3^- reduction, NO_2^- (11). Here, such competition experiments are extended to include Fe^{3+} and either NO_3^- or NO_2^-. Results presented here show that, although NO_2^- is responsible for inhibiting Fe^{3+} reduction activity, NO_2^--catalyzed Fe^{2+} oxidation is not a major factor contributing to the observed inhibition pattern.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. putrefaciens* 200 was obtained from D. W. S. Westlake, University of Alberta, Edmonton, Alberta, Canada. Growth medium consisted of a semidefined lactate medium as described previously (10). The electron acceptor competition experiments described below were conducted with batch cultures of *S. putrefaciens* 200 grown in a 1-liter double-jacketed glass reactor vessel. The vessel was fitted with a stainless steel headplate containing a variety of ports designed to accommodate electrode insertion, sample withdrawal, and reagent addition. The temperature was held constant at 30°C by pumping water from a constant-temperature bath through the outer jacket. Dissolved oxygen content and pH were

† Contribution 7963 of Woods Hole Oceanographic Institution.

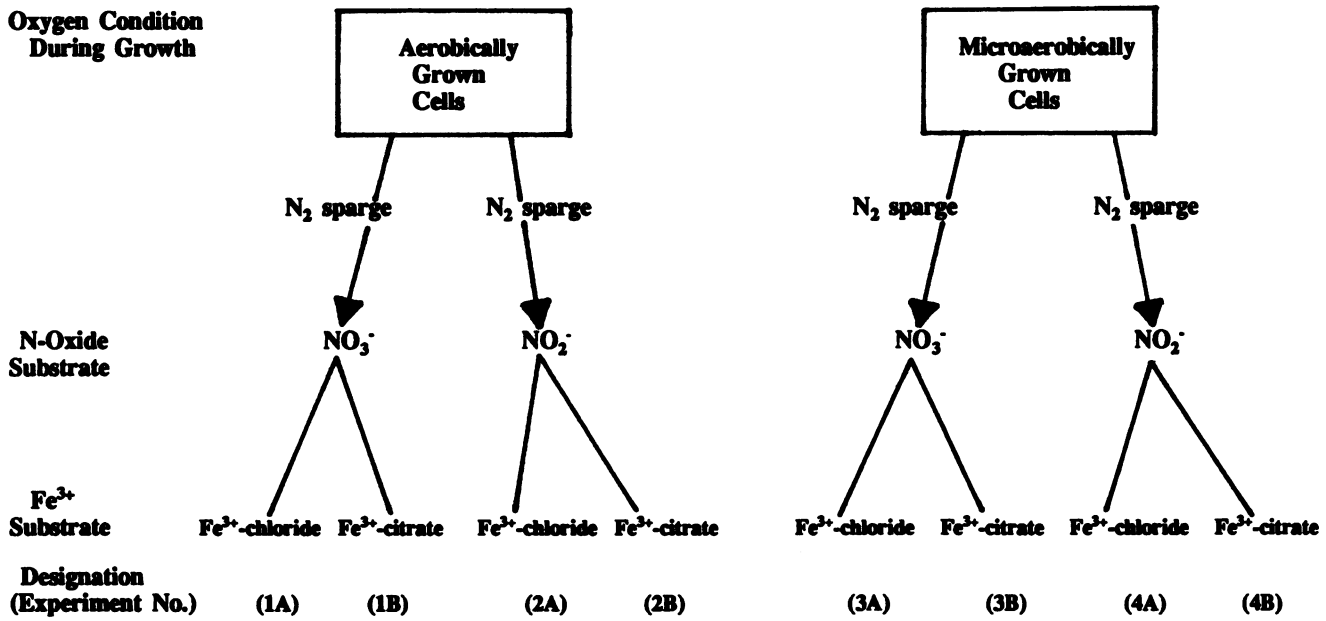

FIG. 1. Experimental design of Fe^{3+}-nitrogen oxide electron acceptor competition experiments (see Materials and Methods for a detailed description).

monitored with an O_2 sensor (Ingold Electrodes, Inc.) and pH probe (Orion Research, Inc.), respectively. Compressed air and high-purity N_2 (g) were introduced through glass spargers inserted through the headplate. The culture pH (7.0 ± 0.1) was maintained by manual injection of either 10 N NaOH or 10 N H_2SO_4. Agitation was accomplished by means of a magnetic stir bar.

Experimental design. The experimental procedure followed during the electron acceptor competition experiments is given schematically in Fig. 1. Two sets of kinetic experiments, differing solely in the dissolved oxygen concentration maintained during batch growth, were conducted to determine the effects of NO_3^- and NO_2^- on the Fe^{3+} reduction activity of *S. putrefaciens* 200. In the first set, cells were grown in batch culture under highly aerobic conditions (>90% air-saturated medium throughout the growth period), whereas in the second set cells were grown in batch culture under microaerobic conditions (<1% air-saturated medium during the last 4 h of growth). Four electron acceptor competition experiments were subsequently conducted with the batch cultures grown under either oxygen condition; equimolar concentrations of a nitrogen oxide (either KNO_3 [15 mM, except where noted] or KNO_2 [3 mM]) and an Fe^{3+} substrate (either Fe^{3+}-chloride or Fe^{3+}-citrate) were sequentially added to each batch, and the concentrations of Fe^{2+} and NO_2^- were monitored for a period of 60 to 80 min after electron acceptor addition. Each electron acceptor was added from a freshly prepared stock solution. Fe^{3+}-chloride and Fe^{3+}-citrate stocks were neutralized with 10 N NaOH before addition. To determine the relative contribution of NO_2^--catalyzed chemical reoxidation of bacterially produced Fe^{2+} to the apparent inhibition of Fe^{3+} reduction activity, each of the four electron acceptor competition experiments was run in duplicate. In the first run, bacterially produced Fe^{2+} was allowed to react chemically with the nitrogen oxides (and their reduced forms, if any); aliquots were withdrawn and immediately tested for the presence of Fe^{2+} and NO_2^- (see Chemical Analyses, below). In the second run, an in situ ferrozine assay (see Chemical Analyses, below) was used to detect bacterially produced Fe^{2+} (i.e., before NO_2^--catalyzed Fe^{2+} oxidation could occur).

Growth was initiated in each batch by adding 2.0 ml of a dense overnight bacterial culture to the growth medium and monitored by periodic measurement of the optical density at 600 nm. When highly aerobic conditions were required during growth, compressed air (filter sterilized) was bubbled in vigorously throughout the growth period; when microaerobic conditions were required during growth, high-purity N_2 (g) and compressed air were trickled in at low rates to maintain dissolved oxygen concentrations at less than 1% air saturation. When a target optical density was reached (optical density at 600 nm of 0.25), chloramphenicol was added to 0.25 mM. Cultures grown under microaerobic conditions were immediately sparged with N_2 to avoid possible inactivation of the ferrireductase, nitrate reductase, or nitrite reductase activity by O_2 (5). A separate experiment was conducted to determine the O_2 utilization rate of cells grown under microaerobic conditions. For cultures grown under highly aerobic conditions, O_2 utilization rates were determined immediately after chloramphenicol addition by monitoring the time-dependent oxygen profile for 5 min. Residual oxygen was subsequently purged from the reactor vessel with high-purity N_2 (g).

The Fe^{2+} and NO_2^- production rates detected in the electron acceptor competition experiments were compared to those rates detected in a series of biotic control experiments. The biotic control experiments consisted of measuring the rate at which O_2, NO_3^-, NO_2^-, Fe^{3+}-citrate, and Fe^{3+}-chloride were reduced when each was provided as the sole electron acceptor to batch cultures grown under either oxygen condition. In the cases of O_2, NO_3^- (microaerobically grown cells only), and NO_2^-, the reduction rates were determined by measuring the rate at which each electron acceptor was depleted. In the case of NO_3^- reduction by aerobically grown cells, the NO_3^- reduction rate was determined by measuring the rate of both NO_3^- depletion and

NO_2^- production. In the cases of Fe^{3+}-chloride and Fe^{3+}-citrate, the reduction rates were determined by measuring the rate of Fe^{2+} production. Anaerobic conditions were maintained throughout the time course of each experiment. Total cellular protein was determined by the method of Lowry et al. (7).

To determine the rate of abiotic reduction of each electron acceptor, Fe^{3+}-citrate, Fe^{3+}-chloride, NO_3^-, and NO_2^- were added to separate 1-liter batches of autoclaved, N_2-sparged growth medium and the concentration of Fe^{2+} or NO_2^- was monitored for a period of 60 to 90 min.

To examine more closely the ability of NO_3^- and NO_2^- to act as chemical oxidants of Fe^{2+}, two series of abiotic oxidation control experiments were conducted. In the first series, 50 μM Fe^{2+} (as $FeCl_2 \cdot 4H_2O$) and either KNO_3 (15 mM) or KNO_2 (3 mM) were added to 1-liter batches of autoclaved, N_2-sparged growth medium (30°C) and the concentration of Fe^{2+} was monitored for a period of 60 to 90 min. In the second series of abiotic oxidation control experiments, an Fe^{3+} substrate (either Fe^{3+}-chloride [15 mM] or Fe^{3+}-citrate [15 mM]) was included in the autoclaved, 1-liter batch of N_2-sparged growth medium along with 50 μM Fe^{2+} and a nitrogen oxide (either KNO_3 [15 mM] or KNO_2 [3 mM]) and the concentration of Fe^{2+} was monitored for a period of 60 to 90 min.

Chemical analyses. (i) Iron. Each electron acceptor competition experiment was run in duplicate. In the first run, the electron acceptors (and their reduced forms, if any) were allowed to react chemically in the liquid culture. Samples were withdrawn at preselected time intervals, and the Fe^{2+} concentration was monitored by the ferrozine extraction method (17, 18). In the second run, ferrozine (to 8 mM) was added directly to the liquid culture prior to electron acceptor addition. This technique permitted detection of Fe^{2+} production in situ and was used to eliminate the effects of chemical reoxidation of Fe^{2+} by NO_2^-. Previous studies demonstrated that the magenta Tris complex between Fe^{2+} and ferrozine forms on a time scale faster than that of Fe^{2+} reoxidation (2, 17). When the in situ ferrozine assay was used, samples were withdrawn at preselected time intervals and immediately placed on ice. Each sample was centrifuged at 12,000 × *g* for 2 min at 4°C, and the A_{562} was measured. The absorbance of the ferrozine-Fe^{2+} complex ($\varepsilon_{562} = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$) was measured on a Beckman DU-7 spectrophotometer.

(ii) Nitrate. For determination of NO_3^-, 1-ml samples were withdrawn at preselected time intervals and microfuged at 12,000 × *g* for 2 min at 4°C and the supernatant was immediately placed at −20°C. NO_3^- concentrations were determined with an Alpkem RFA/2 Autoanalyzer (Alpkem Corp., Clackamas, Ore.).

(iii) Nitrite. For determination of NO_2^-, samples were withdrawn at preselected time intervals and immediately quenched in a sulfanilic acid–*N*-1-naphthylethylene-diamine dihydrochloride solution (8). Each sample was held at room temperature for 10 min to allow full color development. Absorbance measurements (550 nm) were made on a Beckman DU-7 spectrophotometer ($\varepsilon_{550} = 1.9 \times 10^4\ M^{-1}\ cm^{-1}$).

RESULTS

Biotic controls. The rates of bacterially catalyzed electron transport to O_2, NO_3^-, NO_2^-, Fe^{3+}-citrate, and Fe^{3+}-chloride are listed in Table 1. The rates reported in Table 1 were corrected for abiotic electron acceptor reduction by subtracting previously measured abiotic reduction rates (micromolar per minute) (NO_3^-, 0.00; NO_2^-, 0.03; Fe^{3+}-chloride, 0.03; Fe^{3+}-citrate, 0.20) from the biotic reduction rate. The corrected rates were then normalized on a per-mole electron basis by assuming that a 4-mol electron transfer was required to reduce each mole of O_2, a 2-mol electron transfer was required to reduce each mole of NO_3^- or NO_2^-, and a 1-mol electron transfer was required to reduce each mole of Fe^{3+}. After growth under highly aerobic conditions, *S. putrefaciens* 200 expressed low-rate NO_3^- and NO_2^- reductase activities and, as previously reported (1), low-rate cytochrome oxidase and Fe^{3+} reductase activities. After growth under microaerobic conditions, each reductase activity was expressed at a greatly elevated rate. The derepression factor determined for electron transfer to either O_2 or NO_3^- was approximately 2, whereas the derepression factor determined for electron transfer to either Fe^{3+}-chloride or Fe^{3+}-citrate was approximately 20. The derepression factor determined for electron transfer to NO_2^- was approximately 9. Irrespective of the dissolved O_2 concentration maintained during batch growth, the rate of electron transfer to Fe^{3+}-citrate was approximately four- to fivefold greater than the rate of electron transfer to Fe^{3+}-chloride. Aerobically grown cells stoichiometrically converted NO_3^- to NO_2^- (data not shown). Since NO_2^- was toxic to aerobically grown cells at concentrations greater than 4 mM (data not shown), NO_2^- was provided at a final concentration of 3 mM during Fe^{3+}-NO_2^- competition experiments.

TABLE 1. Rates of electron transfer to O_2, NO_3^-, NO_2^-, Fe^{3+}-citrate, and Fe^{3+}-chloride by whole-cell preparations of *S. putrefaciens* 200

Electron acceptor	Electron transfer rate after growth at:		Derepression factor[d]
	High O_2 tension (% of electron transfer rate to O_2)[a,b]	Low O_2 tension (% of electron transfer rate to O_2)[a,c]	
O_2	1,290 (100)	2,636 (100)	2
NO_3^-	101[e] (8)	215 (8)	2
NO_2^-	20 (2)	184 (6)	9
Fe^{3+}-citrate	57 (5)	1,054 (40)	19
Fe^{3+}-chloride	12 (1)	256[f] (10)	21

[a] Rates are expressed as nanomolar electrons per milligram of protein per minute.
[b] High O_2 growth is 90 to 100% air-saturated medium during growth.
[c] Low O_2 growth is 1% air-saturated medium during the last 4 h of growth.
[d] Derepression factor is the ratio of the rate of electron transfer after growth under microaerobic conditions to the rate of electron transfer after growth under highly aerobic conditions.
[e] Rate of NO_2^- production or NO_3^- depletion.
[f] Initial rate of Fe^{3+}-chloride reduction.

Fe^{3+}-nitrogen oxide electron acceptor competition experiments using aerobically grown cells. Results of the Fe^{3+}-nitrogen oxide competition experiments using aerobically grown cells are summarized in Table 2. The bacterially catalyzed rates reported in Table 2 were corrected for abiotic electron acceptor reduction (see text above for values) before being normalized on a per-mole electron basis. Aerobically grown cells reduced NO_3^- and Fe^{3+}-chloride simultaneously (competition experiment 1A). NO_2^- production rates were not affected by the presence of equimolar Fe^{3+}-chloride. However, Fe^{2+} production rates were inhibited approximately 75% by the presence of equimolar NO_3^-. The inhibited Fe^{2+} production rate detected by using the in situ ferrozine assay was nearly identical to that rate detected when Fe^{2+} chemical interaction was allowed. This finding

TABLE 2. Results of Fe^{3+}-nitrogen oxide electron acceptor competition experiments

Growth conditions	Electron acceptor competition expt	NO_2^- production rate[a,b]		Fe^{2+} production rate[a,b]			
		Chemical interaction allowed	% Inhibition[c]	In situ ferrozine assay	% Inhibition[c]	Chemical interaction allowed	% Inhibition[c]
Aerobic	1A. Fe^{3+}-chloride-NO_3^-	87 (1.00)	0	3 (1.00)	75	3 (0.99)	75
	Fe^{3+}-chloride			12 (1.00)			
	NO_3^-	81 (1.00)					
	1B. Fe^{3+}-citrate-NO_3^-	84 (1.00)	5	48 (0.99)	16	41 (1.00)	28
	Fe^{3+}-citrate			57 (1.00)			
	NO_3^-	81 (1.00)					
	2A. Fe^{3+}-chloride-NO_2^-			4 (1.00)	67	3 (0.98)	75
	Fe^{3+}-chloride			12 (1.00)			
	NO_2^-						
	2B. Fe^{3+}-citrate-NO_2^-			37 (1.00)	26	39 (0.99)	22
	Fe^{3+}-citrate			50 (0.99)			
	NO_2^-						
Microaerobic	3A. Fe^{3+}-chloride-NO_3^-			250 (1.00)	2	134 (0.97)	46
	Fe^{3+}-chloride			256 (1.00)			
	NO_3^-						
	3B. Fe^{3+}-citrate-NO_3^-			987 (1.00)	6	1,034 (1.00)	2
	Fe^{3+}-citrate			1,054 (1.00)			
	NO_3^-						
	4A. Fe^{3+}-chloride-NO_2^-	193 (0.90)	0	49 (1.00)	81	21 (0.98)	92
	Fe^{3+}-chloride			256 (1.00)			
	NO_2^-	184 (0.97)					
	4B. Fe^{3+}-citrate-NO_2^-	178 (0.95)	3				
	Fe^{3+}-citrate			ND[d]	ND		
	NO_2^-	184 (0.97)					

[a] Rates are expressed as nanomolar electrons per milligram of protein per minute (correlation coefficient in parentheses).
[b] The results given for electron acceptor competition experiments 3A and 4A are the initial rates of Fe^{3+}-chloride reduction and the results for experiments 4A and 4B are the rates of NO_2^- depletion.
[c] Percent inhibition = [1 − (reduction rate in presence of competing electron acceptor/reduction rate in absence of competing electron acceptor)] × 100.
[d] ND, not determined.

agreed with the results of previous abiotic Fe^{2+}-NO_3^- oxidation controls (data not shown), which demonstrated that NO_3^- oxidized Fe^{2+} at relatively slow rates (0.06 μM min^{-1}, $r^2 = 0.97$). When Fe^{3+}-citrate was substituted for Fe^{3+}-chloride in an otherwise identical set of competition experiments with NO_3^- (competition experiment 1B), a similar inhibition pattern was observed. Aerobically grown cells reduced Fe^{3+}-citrate and NO_3^- simultaneously. NO_2^- production rates were not affected by the presence of equimolar Fe^{3+}-citrate; however, Fe^{2+} production rates were inhibited approximately 16 to 28% by the presence of equimolar NO_3^-. The inhibited Fe^{2+} production rate detected by using the in situ ferrozine assay was nearly identical to that rate detected when Fe^{2+} chemical interaction was allowed.

In the Fe^{3+}-NO_3^- competition experiments described above, NO_2^- was produced and therefore was available to act as an inhibitor of Fe^{3+} reduction activity. NO_2^- was subsequently substituted for NO_3^-, and an otherwise identical set of competition experiments was conducted. In both competition experiments (Fe^{3+}-chloride-NO_2^- [experiment 2A] and Fe^{3+}-citrate-NO_2^- [experiment 2B]), Fe^{2+} production rates detected by using the in situ ferrozine assay were nearly identical to those rates detected when Fe^{2+} chemical interaction was allowed. This finding agreed with the result of previous abiotic Fe^{2+}-NO_2^- oxidation controls (data not shown), which demonstrated that NO_2^- oxidized Fe^{2+} at relatively slow rates (0.05 μM min^{-1}, $r^2 = 0.95$). In the case of Fe^{3+}-chloride (experiment 2A), Fe^{2+} production rates were inhibited approximately 67 to 75% by the presence of NO_2^- alone; results from competition experiment 1A (see above) demonstrated that Fe^{2+} production rates were inhibited to a similar extent by the presence of both NO_2^- and NO_3^-. An identical inhibition pattern was observed in the case of Fe^{3+}-citrate (experiment 2B): Fe^{2+} production rates were inhibited approximately 22 to 26% by the presence of NO_2^- alone, whereas results from competition experiment 1B (see above) demonstrated that Fe^{2+} production was inhibited to a similar extent by the presence of both NO_2^- and NO_3^-. The inhibitory effects of NO_2^- and NO_3^- on the Fe^{3+} reduction activity of aerobically grown cells were much less pronounced when Fe^{3+}-citrate was used as a competing electron acceptor.

Fe^{3+}-nitrogen oxide electron acceptor competition experiments using microaerobically grown cells. Results of Fe^{3+}-nitrogen oxide competition experiments using microaerobically grown cells are summarized in Table 2. The rates reported in Table 2 were corrected for abiotic electron acceptor reduction before being normalized on a per-mole electron basis. The inhibitory effects of NO_3^- on the reduction of either Fe^{3+}-chloride (experiment 3A) or Fe^{3+}-citrate (experiment 3B) were relatively small (2% and 6% inhibition, respectively). In the case of Fe^{3+}-citrate, Fe^{2+} production rates detected after Fe^{2+}-NO_3^- chemical interaction was allowed were nearly identical to those rates detected by using the in situ ferrozine assay. These results agreed with the results of previous abiotic Fe^{2+}-NO_3^- oxidation controls (see above), which demonstrated that NO_3^- oxidized Fe^{2+} at relatively slow rates. However, in the case of Fe^{3+}-chloride and NO_3^- (experiment 3A), Fe^{2+} production rates

detected after Fe^{2+}-NO_3^- chemical interaction was allowed were only one-half as fast as those rates detected by using the in situ ferrozine assay. Reasons for this rate discrepancy remain unclear.

Microaerobically grown cells also reduced Fe^{3+} and NO_2^- simultaneously. NO_2^- reduction rates were unaffected by the presence of either Fe^{3+}-chloride (experiment 4A) or Fe^{3+}-citrate (experiment 4B). However, Fe^{3+}-chloride reduction (experiment 4A) was severely inhibited by the presence of NO_2^-: Fe^{2+} production rates were inhibited approximately 81% when the in situ ferrozine assay was used and were inhibited approximately 92% after Fe^{2+} and NO_2^- were allowed to interact chemically. Results of the Fe^{3+}-citrate–NO_2^- competition experiment (experiment 4B) were difficult to interpret (data not shown) because Fe^{3+}-citrate was provided at below-saturation levels (3 mM).

DISCUSSION

The inhibitory effects of NO_3^- and NO_2^- on the Fe^{3+} reduction activity of *S. putrefaciens* 200 were examined in a series of electron acceptor competition experiments. The effects of NO_3^- and NO_2^- on the Fe^{3+} reduction of both aerobically and microaerobically grown cells appeared to follow a consistent pattern. In the presence of Fe^{3+} and either NO_3^- or NO_2^-, dissimilatory Fe^{3+} and nitrogen oxide reduction occurred simultaneously. Nitrogen oxide reduction was not affected by the presence of Fe^{3+}, suggesting that *S. putrefaciens* 200 expressed a set of at least three physiologically distinct terminal reductases that served as electron donors to NO_3^-, NO_2^-, and Fe^{3+}. However, Fe^{3+} reduction was partially inhibited by the presence of either NO_3^- or NO_2^-. Two lines of evidence suggested that the apparent NO_3^- inhibition of Fe^{3+} reduction activity was due to a factor other than NO_3^--catalyzed chemical reoxidation of bacterially produced Fe^{2+}: (i) results from abiotic Fe^{2+}-NO_3^- oxidation controls, which demonstrated that chemical oxidation of Fe^{2+} by NO_3^- occurred at negligible rates; and (ii) the observation that the Fe^{2+} production rates detected when bacterially produced Fe^{2+} was allowed to interact chemically with NO_3^- were nearly identical to those rates detected when an in situ ferrozine assay was used to minimize NO_3^--catalyzed Fe^{2+} reoxidation. However, the apparent decrease in the Fe^{2+} production rate was significantly less than that expected if NO_3^- were acting as a competitive inhibitor of electron transport to Fe^{3+}. In addition, the presence of Fe^{3+} did not appear to affect the rate at which aerobically grown cells reduced NO_3^- (produced NO_2^-). Although partial NO_3^- inhibition of Fe^{3+} reduction activity could not be ruled out, these results suggested that NO_3^- acted neither as a chemical oxidant of bacterially produced Fe^{2+} nor as a competitive inhibitor of electron transport to Fe^{3+}.

In the presence of Fe^{3+} and NO_3^-, aerobically grown cells produced Fe^{2+} and NO_2^- simultaneously. Therefore, NO_2^- was available in the Fe^{3+}-NO_3^- competition experiments to act either as a chemical oxidant of bacterially produced Fe^{2+} or as a competitive inhibitor of electron transport to Fe^{3+}. The former explanation for the apparent NO_3^- inhibition of Fe^{3+} reduction activity seemed unlikely in light of three observations: (i) the relatively slow rate at which NO_2^- chemically oxidized Fe^{2+} during abiotic Fe^{2+}-NO_2^- oxidation controls, (ii) the apparent inability of high levels of bacterially produced Fe^{2+} to affect the rate at which NO_2^- was produced during Fe^{3+}-NO_3^- competition experiments, and (iii) the results of subsequent Fe^{3+}-NO_2^- competition experiments which demonstrated that the Fe^{2+} production rates detected by using an in situ ferrozine assay were nearly identical to those rates detected when Fe^{2+}-NO_2^- chemical interaction was allowed. These data suggested that the apparent NO_2^- inhibition of Fe^{3+} reduction activity was due to a factor other than NO_2^- acting as a chemical oxidant of bacterially produced Fe^{2+}. Whether NO_2^- acted as a competitive inhibitor of electron transport to Fe^{3+} could not be assessed accurately with these data. However, the apparent decrease in Fe^{3+} reduction activity in the presence of both NO_3^- and NO_2^- was approximately equal to the apparent decrease in Fe^{3+} reduction activity in the presence of NO_2^- alone. These results suggested that bacterially produced NO_2^- was responsible for inhibiting electron transport to Fe^{3+}.

Obuekwe and coworkers (11) previously used *S. putrefaciens* 200 as a model iron-reducing microorganism to study the effect of NO_3^- on microbial Fe^{3+} reduction. Batch cultures were grown aerobically and either preinduced (via 90 min of anaerobic incubation in the presence of NO_3^-) or not induced for expression of NO_3^- reductase activity. Preinduced cells produced NO_2^- immediately, whereas uninduced cells produced NO_2^- only after a 2-h lag period. Presumably the uninduced cells were induced for NO_3^- reductase expression during this lag period (protein synthesis was allowed during these experiments). This finding was different from that observed in the present study, in which NO_2^- production was detected without preinduction of NO_3^- reductase activity; after growth under highly aerobic conditions, *S. putrefaciens* 200 expressed both low-rate NO_3^- reductase and low-rate NO_2^- reductase activities, whereas after growth under microaerobic conditions, *S. putrefaciens* 200 expressed greatly elevated rates of each reductase activity. The reason for the observed difference in the growth condition required for NO_3^- reductase expression remains unclear. However, in the present study, lactate was used as the principal carbon and energy source for growth, whereas Obuekwe and coworkers (11) used β-glycerophosphate for growth and subsequently used lactate during the NO_3^- reductase preinduction period and Fe^{3+}-NO_3 competition experiments. The observed lag in NO_2^- production may have been the lag time required for expression of enzymes involved in either lactate oxidation or NO_3^- reduction.

In their Fe^{3+}-NO_3^- competition experiments, Obuekwe and coworkers (11) reported that Fe^{2+} production was inhibited only after an initial 3-h incubation period. During this initial incubation period, Fe^{2+} and NO_2^- were produced simultaneously. During the last 4 h, a marked decrease in the production of both Fe^{2+} and NO_2^- was observed. In several experiments, Fe^{2+} and NO_2^- were depleted during this time period. The investigators hypothesized that bacterially produced NO_2^- was chemically reoxidizing Fe^{2+} at a rate faster than the rate of bacterially catalyzed Fe^{2+} production. Fe^{3+}-NO_2^- competition experiments to examine this possibility were not conducted. In addition, because protein synthesis was allowed during these experiments, it was possible that NO_2^- reductase activity was expressed during the 3-h anaerobic lag period which immediately preceded the onset of the observed inhibitory effect. Since *S. putrefaciens* 200 is capable of anaerobic growth by using NO_3^-, NO_2^-, or Fe^{3+} as the sole terminal electron acceptor (unpublished data), the observed inhibitory effects of NO_3^- and NO_2^- on Fe^{3+} reduction may be due to the preferential shuttling of respiratory electrons to NO_2^- via a newly synthesized high-rate NO_2^- reductase. In the present study, NO_2^-

reductase activity was enhanced nearly 10-fold via growth under microaerobic conditions. Although the Fe^{2+} reoxidation theory of Obuekwe and coworkers (11) could be supported by their Fe^{3+}-NO_3^- competition data, the inhibition theory proposed in the present study cannot be ruled out. Results from the present study indicate that NO_3^- inhibition of electron transport to Fe^{3+} is caused by a preferential shuttling of respiratory electrons to bacterially produced NO_2^-; Fe^{3+} may serve as a secondary sink for excess electrons after NO_2^- reductase activity is fully saturated. The electron transport pathways to Fe^{3+} and NO_2^- may share a common respiratory chain component (i.e., branch point) that is poised to deliver electrons to the more oxidizing electron acceptor. Genetic and biochemical experiments are currently under way to examine this possibility.

The Fe^{3+} reduction system of *S. putrefaciens* 200 is thought to contain low-rate and high-rate components (1). Results from the present study confirmed those findings and demonstrated that the NO_3^- and NO_2^- reduction systems of *S. putrefaciens* 200 also consist of low-rate and high-rate components. The inhibition patterns observed during Fe^{3+}-NO_3^- and Fe^{3+}-NO_2^- electron acceptor competition experiments suggest, but do not prove, that *S. putrefaciens* 200 expresses a set of at least three physiologically distinct terminal reductases that serve as electron donors for NO_3^-, NO_2^-, and Fe^{3+}. It is proposed that electron transport to Fe^{3+} and NO_3^- proceeds via two physiologically uncoupled pathways, whereas electron transport to Fe^{3+} and NO_2^- proceeds via a shared respiratory chain component (branch point) that is poised to transfer electrons to the more oxidizing (or readily available) electron acceptor. In the presence of Fe^{3+} and NO_2^-, Fe^{3+} serves as a secondary sink for excess respiratory electrons when the overall rate of anaerobic electron transport is limited by NO_2^- reductase activity.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Mellon Foundation and the Powell Foundation and by grant no. OCE-9023943 from the National Science Foundation.

I thank Jane Peterson for help in manuscript preparation and Zofia Mlodzinska for performing the NO_3^- determinations. I also thank Ed DeLong, Ken Nealson, and Mary Lidstrom for technical advice and critical review of the manuscript.

REFERENCES

1. **Arnold, R. G., T. J. DiChristina, and M. R. Hoffmann.** 1986. Inhibitor studies of dissimilative Fe^{3+} reduction by *Pseudomonas* sp. strain 200 ("*Pseudomonas ferrireductans*"). Appl. Environ. Microbiol. **52**:281–289.
2. **Arnold, R. G., M. R. Hoffmann, T. J. DiChristina, and F. W. Picardal.** 1990. Regulation of dissimilative Fe^{3+} reduction in *Shewanella putrefaciens*. Appl. Environ. Microbiol. **56**:2811–2817.
3. **Berner, R. A.** 1964. Iron sulfides formed from aqueous solution at low temperature and atmospheric pressure. J. Geol. **72**:293–306.
4. **Brons, H. J., R. H. Wilfred, and A. J. B. Zehnder.** 1991. Ferrous iron dependent nitric oxide production in NO_3^- reducing cultures of *Escherichia coli*. Arch. Microbiol. **155**:341–347.
5. **Gorby, Y. A., and D. R. Lovley.** 1991. Electron transport in the dissimilatory iron reducer, GS-15. Appl. Environ. Microbiol. **57**:867–870.
6. **Lascelles, J., and K. A. Burke.** 1978. Reduction of ferric iron by L-lactate and DL-glycerol-3-phosphate in membrane preparations from *Staphylococcus aureus* and interactions with the nitrate reductase system. J. Bacteriol. **134**:585–589.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
8. **Montgomery, H. A. C., and J. F. Dymock.** 1961. The determination of nitrite in water. Analyst (London) **86**:414–416.
9. **Myers, C. R., and K. H. Nealson.** 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science **240**:1319–1321.
10. **Obuekwe, C. O., and D. W. S. Westlake.** 1982. Effect of medium composition on cell pigmentation, cytochrome content and ferric iron reduction in a *Pseudomonas* sp. isolated from crude oil. Can. J. Microbiol. **28**:989–992.
11. **Obuekwe, C. O., D. W. S. Westlake, and F. D. Cook.** 1981. Effect of nitrate on reduction of ferric iron by a bacterium isolated from crude oil. Can. J. Microbiol. **27**:692–697.
12. **Ottow, J. C. G.** 1968. Evaluation of iron-reducing bacteria in soil and the physiological mechanism of iron reduction in *Aerobacter aerogenes*. Z. Allg. Mikrobiol. **8**:441–443.
13. **Ottow, J. C. G.** 1970. Selection, characterization and iron-reducing capacity of nitrate reductaseless (*nit*$^-$) mutants from iron-reducing bacteria. Z. Allg. Mikrobiol. **10**:55–62.
14. **Ponnamperuma, F. N., E. Tianco, and T. Loy.** 1967. Redox equilibria in flooded soils. 1. The iron hydroxide systems. Soil Sci. **103**:374–382.
15. **Semple, K. M., J. L. Doran, and D. W. S. Westlake.** 1989. DNA relatedness of oil field isolates of *Shewanella putrefaciens*. Can. J. Microbiol. **35**:925–931.
16. **Semple, K. M., and D. W. S. Westlake.** 1987. Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. Can. J. Microbiol. **33**:366–371.
17. **Sørenson, J.** 1982. Reduction of ferric iron in anaerobic, marine sediment and interaction with reduction of nitrate and sulfate. Appl. Environ. Microbiol. **43**:319–324.
18. **Stookey, L. L.** 1970. Ferrozine: a new spectrophotometric reagent for iron. Anal. Chem. **42**:779–782.
19. **Stumm, W., and J. J. Morgan.** 1981. Aquatic chemistry. Wiley Interscience, New York.
20. **Troshanov, E. P.** 1968. Iron- and manganese-reducing microorganisms of ore-containing lakes of the Karelian isthmus. Mikrobiologiya **37**:786–791.