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Anaerobic Electron Acceptor Chemotaxis in *Shewanella putrefaciens*

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Shewanella putrefaciens **MR-1 can grow either aerobically or anaerobically at the expense of many different electron acceptors and is often found in abundance at redox interfaces in nature. Such redox interfaces are often characterized by very strong gradients of electron acceptors resulting from rapid microbial metabolism. The coincidence of *S. putrefaciens* abundance with environmental gradients prompted an examination of the ability of MR-1 to sense and respond to electron acceptor gradients in the laboratory. In these experiments, taxis to the majority of the electron acceptors that *S. putrefaciens* utilizes for anaerobic growth was seen. All anaerobic electron acceptor taxis was eliminated by the presence of oxygen, nitrate, nitrite, elemental sulfur, or dimethyl sulfoxide, even though taxis to the latter was very weak and nitrate and nitrite respiration was normal in the presence of dimethyl sulfoxide. Studies with respiratory mutants of MR-1 revealed that several electron acceptors that could not be used for anaerobic growth nevertheless elicited normal anaerobic taxis. Mutant M56, which was unable to respire nitrite, showed normal taxis to nitrite, as well as the inhibition of taxis to other electron acceptors by nitrite. These results indicate that electron acceptor taxis in *S. putrefaciens* does not conform to the paradigm established for *Escherichia coli* and several other bacteria. Carbon chemotaxis was also unusual in this organism: of all carbon compounds tested, the only positive response observed was to formate under anaerobic conditions.**

Bacterial aerotaxis and carbon chemotaxis have been known for many years (2, 3, 6, 10, 13), but detailed studies of the mechanisms underlying electron acceptor chemotaxis have only recently been initiated (4, 5, 11, 12, 23, 24, 27–29). In contrast to carbon chemotaxis, which is well understood in *Escherichia coli* and is mediated via either widely distributed (15) methyl-accepting chemotaxis proteins or the phosphotransferase sugar transport system (2, 3, 6, 13), electron acceptor taxis remains unexplained mechanistically. Neither the primary signals nor the acceptors of those signals have been identified with certainty. Aerotaxis as well as the other electron acceptor taxes is apparently a methyl-accepting chemotaxis protein- and phosphotransferase-independent system (11–13, 27–29). Mutant *E. coli* and *Salmonella typhimurium* strains deficient in methyl transfer show normal aerotaxis (9, 21), which is proposed to be mediated through the proton motive force via a mechanism termed the protometer (31). The accepted paradigm is that electron transport resulting from the metabolism of a given compound is required for taxis to it, although the exact nature of the receptor(s) remains unknown. It is hypothesized that aerotaxis in *Bacillus cereus* is mediated by the proton motive force as in the enteric bacteria (12). *Rhodobacter sphaeroides* is strongly aerotactic after anaerobic growth in the dark, and the mechanism of its chemotactic sensing is not yet understood (5, 23, 24, 30). Experiments with inhibitors suggested that, as with other bacteria, aerotaxis in *R. sphaeroides* requires active electron transport and not merely the binding of oxygen to a terminal cytochrome (5).

Redox interfaces in nature take many forms in addition to the well-known oxic-anoxic transition zone. These include redox-clines where nitrate, nitrite, Mn(IV), Fe(III), and sulfate are consumed, usually in the order of oxidative energy; i.e., the electron acceptor with the highest redox potential is preferentially used first. An excellent example of this is the suboxic zone of the Black Sea, where successive redox interfaces occur at depths of 50 m (where oxygen disappears) to about 100 m (where the sulfide concentration begins to increase [18]). Similar phenomena are well-known in virtually every anoxic sediment in both lacustrine and marine environments, often occurring over centimeters or millimeters rather than meters. In such environments, one might ask whether the ability to respond to and move toward a potentially useful electron acceptor might be advantageous to a given bacterium. Because *Shewanella putrefaciens* can utilize more than a dozen different respiratory electron acceptors (16), and because it is often found in high numbers at redox interfaces (7, 18), we examined its chemotactic behavior to these compounds. In this paper, we describe some of the chemotactic properties of *S. putrefaciens* MR-1 and derived mutants under both aerobic and anaerobic conditions. The chemotactic behavior observed is suggestive of an organism well adapted to existence as a respiratory anaerobe.

MATERIALS AND METHODS

Bacterial strains. *S. putrefaciens* MR-1 is a facultatively anaerobic, gram-negative bacterium that is motile by a single polar flagellum. It was isolated by anaerobic enrichment for manganese-reducing microbes from the sediments of Oneida Lake, N.Y. (16). In addition to oxides of iron and manganese, this organism can utilize nitrate, nitrite, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), thiosulfate, fumarate (16), and elemental sulfur (19) as terminal electron acceptors for respiration. Respiratory mutants (except MetrA) derived from MR-1 were obtained by using the mutagen ICR 191 (Sigma) as described previously (25) and screened for anaerobic growth with various terminal electron acceptors. MRN36 is a motile, pleiotropic mutant incapable of anaerobic growth (25). M56A and M56 are motile respiratory mutants at least partially deficient in nitrate and nitrite metabolism (this work). MetrA has a mutation in the *fnr*-like gene of MR-1, is unable to grow on any electron acceptors except oxygen, nitrate or, Mn(IV), and was generated by gene replacement as previously described (26).

Culture conditions. Wild-type *S. putrefaciens* MR-1 and respiratory mutants were maintained aerobically on Luria-Bertani (14) agar. Strain MetrA requires the addition of 8 μg of tetracycline per ml for stability (26). Cells grown aerobically overnight at 30°C in liquid LB medium were routinely used for chemotaxis

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assays. An anaerobic glove chamber (Coy Laboratory Products, Grass Lake, Mich.) with an atmosphere of 10% H_2 (the balance in N_2) was employed for anaerobic culture propagation as well as for chemotaxis assays.

Growth-condition control cultures were incubated for 24 to 36 h in the anaerobic chamber at an ambient temperature of ca. 23°C in either liquid LB or M1 medium (16) (supplemented with 0.2 g of peptone and 0.1 g of yeast extract per liter). Lactate (20 mM) was added as an electron donor and carbon source, and 10 mM nitrate, 20 mM TMAO, or 20 mM fumarate was added as a respiratory electron acceptor. Cells were also grown anaerobically in liquid LB lacking an added electron donor or acceptor. Cell densities were equalized to that of an aerobic culture prior to performance of chemotaxis assays. MR-1 and mutant anaerobic motility controls were incubated at the same cell densities as those in the standard plate assay for 10 h in liquid LB containing 3 μg of chloramphenicol per ml and electron acceptors at 10 mM.

Chemotaxis assays. An agar plate method similar to that described by Armitage and others (4, 5, 23, 24) was used to provide a qualitative assessment of bacterial chemotaxis to substrates. In this assay, cells suspended in LB soft agar were tested for the tendency to accumulate around wells containing carbon substrates or respiratory electron acceptors. Anaerobic trials were performed at ambient temperature in the Coy glove chamber, and aerobic trials (with oxygen as a chemotactic antagonist) were performed as open air incubations.

Chemotaxis plate assays were prepared as follows. Portions (15 ml) of melted 1.5% LB agar containing chloramphenicol (final concentration of 2 or 3 μg/ml) were stirred into 10-ml portions of dense (overnight) bacterial culture in liquid LB pipetted into disposable petri plates (15 by 100 mm), Chemotactic competitors were added from concentrated stocks at this time. The resulting 25-ml bacterial suspensions contained ca. 5×10^8 cells · ml^{-1} in 0.9% agar. Sample wells (usually four per plate) were created by inverting test tubes (12 by 75 mm) as the agar solidified. Test compounds were added to the resulting 0.5-ml capacity wells (100 mM in the standard assay), and the plates were transferred to the anaerobic chamber. Stabilized concentration gradients were thus produced as test compounds diffused from their source wells. The inclusion of chloramphenicol was essential to prevent bacterial growth which otherwise might be inadvertently scored as a tactic effect (5). The growth of *S. putrefaciens* MR-1 is more effectively inhibited by this antibiotic than is energy metabolism, as only 1 to 2 μg · ml^{-1} is sufficient to retard growth for up to 12 h in LB, yet 3 μg · ml^{-1} does not adversely affect aerobic or anaerobic motility as determined by microscopic examination (unpublished data). Plates were scored after 6 to 10 h for accumulations of cells around the wells, which were readily visible with back and side illumination.

Competition between electron acceptors was tested anaerobically. One electron acceptor (the antagonist, generally at 10 mM) was added to the cell suspension prior to agar polymerization in an otherwise standard plate assay. A competing electron acceptor was then added to an assay well at 100 mM, and the plate was incubated as described above. Molecular oxygen was also tested as a chemotactic competitor by the aerobic incubation of assay plates. The minimum electron acceptor concentrations for which the plate assay was effective were determined empirically by the addition of electron acceptors to wells in serial, twofold dilutions.

For reasons that remain unclear, the capillary method of Adler (1) failed to provide a clear indication of *S. putrefaciens* chemotaxis, whereas *E. coli* controls showed reproducible accumulations of cells around the ends of the capillary tubes containing carbon substrates (data not shown). A recently described chemotactic chamber (20) provided quantitative, real-time data and was used to confirm some of the plate assay results.

Nitrate reduction assay. Overnight cultures of wild-type MR-1 or respiratory mutants grown aerobically in liquid LB were diluted 1:2 in fresh LB. Lactate and nitrate were added at 5 mM and 2 mM, respectively, and the cultures were transferred to the anaerobic chamber. Starting with time zero and at 30-min intervals, 1 ml of the samples was collected and passed through 0.2-μm-pore-size nylon membrane syringe filters. After 100-fold dilution of the samples in deionized water, the concentrations of nitrate and nitrite ions were determined by the cadmium reduction method (8) as adapted for flow injection analysis.

In order to test the effect of DMSO on the respiration of nitrate in the wild type (Fig. 1), 10 mM DMSO, 5 mM lactate, and 2 mM nitrate were added to dense bacterial cultures, diluted 1:2 as described above, and their nitrate reduction dynamics was monitored by the cadmium reduction method as described above.

RESULTS

Electron acceptor taxis. The tactic responses of MR-1 cells to individual electron acceptors are summarized in Table 1. In our standardized assay, electron acceptors were tested at 100 mM. Lower electron acceptor concentrations generally produced correspondingly narrower zones of attraction (data not shown). Chemotaxis to nitrate and nitrite was generally most pronounced, showing zones of accumulation of up to several centimeters around the wells, while that to fumarate, DMSO, and TMAO was weaker and that to thiosulfate was weaker still, often barely visible after 8 to 10 h of incubation. Elemental sulfur was difficult to assess by this method because of the formation of precipitates in the medium, while neither Fe(III) citrate nor colloidal Mn(IV) oxide elicited any visible tactic response.

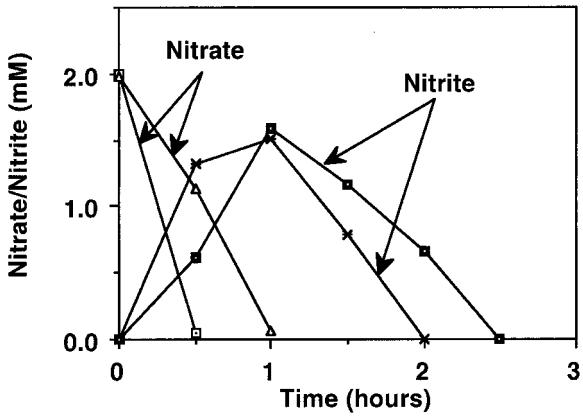

FIG. 1. Effect of 10 mM DMSO on nitrate and nitrite reduction by MR-1. Nitrate (2 mM) was added to a dense cell suspension either containing (△ and ■) or lacking (□ and ×) DMSO. Nitrate consumption and the subsequent production and consumption of nitrite were monitored. Neither nitrate nor nitrite consumption was altered by the presence of DMSO, although chemotaxis to both was inhibited (Table 1).

Lacking an understanding of the diffusion characteristics of electron acceptors in dilute agar or the extent to which cells consume these substrates under assay conditions, it was impossible to determine the actual substrate concentration in that portion of a gradient to which cells responded. Albeit of limited interpretive value, we observed tactic responses to nitrate and nitrite at a concentration as low as 2.5 mM, to fumarate at 6.25 mM, and to TMAO, DMSO, and thiosulfate at 12.5 mM.

Carbon chemotaxis. Carbon and energy sources known to be utilized by MR-1 (17) were tested at 100 mM for chemotactic effects by both the plate assay described here and a spectrophotometric method described previously (20). With the exception of a weakly positive response usually noted for formate under anaerobic conditions, MR-1 failed to display chemotaxis to any carbon source. The carbon substrates tested for which no responses were noted included methanol, ethanol, acetate,

TABLE 1. Chemotaxis and competition between electron acceptors for *S. putrefaciens* MR-1[a]

Attractant[b]	Competitor[c]								
	None	O_2[d]	NO_3^-	NO_2^-	DMSO	S^0	Fumarate	TMAO	$S_2O_3^-$
NO_3^-	+++[e]	−	−	−	−	−	+++	+++	+++
NO_2^-	+++	−	−	−	−	−	+++	+++	+++
Fumarate	++	−	−	−	−	−	−	±	++
TMAO	+	−	−	−	−	−	−	−	±
DMSO	+	−	−	−	−	−	+	±	+
$S_2O_3^{-2}$	±	−	−	−	−	−	−	−	−

[a] Plate migration assays were performed under anaerobic conditions (except in the case where O_2 was the competitor).
[b] Attractants were added to wells at 100 mM.
[c] Competing electron acceptors were added to medium at 10 mM.
[d] Oxygen competition was accomplished by incubation open to the air.
[e] +++, very strong chemotaxis (accumulation of cells up to 1 to 2 cm around the well); ++, strong taxis (accumulation of cells from 0.5 to 1.0 cm); +, visible taxis of less than 0.5 cm; ±, cell accumulation barely visible above the negative background; −, no visible cell accumulation.

TABLE 2. Chemotaxis and antagonism of chemotaxis in *S. putrefaciens* MR-1 and mutants

Strain[a]	Nitrate reduction	Nitrate chemotaxis	Nitrate inhibition of fumarate chemotaxis	Nitrite reduction	Nitrite chemotaxis	Nitrite inhibition of fumarate chemotaxis	Chemotaxis to fumarate, thiosulfate, DMSO, TMAO
MR-1	+	+	+	+	+	+	+
MRN36	+	−	NA[b]	slow	−	NA	−
M56A	+	+	+	very slow	+	+	+
M56	+	+	+	−	+	+	+
MetrA	+	+	+	+	+	+	+

[a] MR-1, wild type; MRN36, nonchemotactic, motile mutant; M56A, M56, and MetrA, motile, respiratory mutants that show normal patterns of chemotaxis and antagonism of chemotaxis.
[b] NA, not applicable.

lactate, propionate, pyruvate, citrate, arginine, serine, cysteine, malate, and glucose. Various combinations of vitamins and amino acids as well as yeast extract and peptone were also tested by plate assay and failed to produce any detectable effect.

Competition between electron acceptors. The results of competition experiments are shown in Table 1. As expected, oxygen, nitrate, and nitrite were strong inhibitors of taxis to other electron acceptors; fumarate, TMAO, and thiosulfate had no effect on chemotaxis to nitrate or nitrite, and thiosulfate did not affect taxis to any of the other electron acceptors. DMSO and elemental sulfur, however, abolished chemotaxis to all other electron acceptors tested, including the more energetically favorable compounds nitrate and nitrite.

The possibility that the inhibition of chemotaxis to other electron acceptors noted in the presence of nitrate, nitrite, DMSO, and elemental sulfur might be due to a general inhibition of cell motility rather than chemotaxis was addressed. Cells incubated in liquid LB with chloramphenicol and these electron acceptors at 10 mM under conditions identical to those for the standard plate assay were microscopically examined. In all cases, the cells retained the appearance of normal motility.

Respiration of nitrate and nitrite in the presence of other electron acceptors. Given that 10 mM DMSO abolished chemotaxis to both nitrate and nitrite, the production and subsequent respiratory consumption of nitrite from 2 mM nitrate were determined for MR-1 in the presence and absence of 10 mM DMSO. These experiments indicated that the addition of DMSO had a minimal effect on the respiration of nitrate or nitrite (Fig. 1). In contrast, as with molecular oxygen, the addition of 10 mM elemental sulfur to cells strongly inhibited both nitrate and nitrite removal (data not shown), as well as chemotaxis to all electron acceptors tested (Table 1).

Chemotaxis of respiratory mutants of MR-1. The chemotactic behavior of several respiratory mutants was not correlated with the ability to reduce or use electron acceptors for growth (Table 2; Fig. 2). While the characterization of other phenotypic aspects of some of these mutants remains incomplete, it is clear that their individual nitrate or nitrite reduction dynamics vary considerably (Fig. 2). Wild-type MR-1 and mutant MetrA depleted nitrate within 1 h and then rapidly consumed the formed nitrite, whereas mutant M56 showed almost no depletion of nitrite after 13 h, as judged by uptake studies (Fig. 2). Mutants M56 and M56A were normally chemotactic towards nitrate and nitrite, although they were deficient in the reduction of these substrates (Fig. 2). MRN36, a motile pleiotropic mutant which cannot grow on any anaerobic electron acceptor (25), showed no taxis to any of the anaerobic electron acceptors, including nitrate and nitrite, which it clearly reduced (although at a somewhat impaired rate [Fig. 2]).

DISCUSSION

Electron acceptor taxis (including aerotaxis) in *E. coli* is thought to occur via a mechanism termed the protometer. The concept central to the protometer hypothesis is that the flow of electrons through an electron transport system is the actual chemotactic signal, sensed either directly or indirectly through resulting changes in the proton motive force. In this manner, cells can choose between chemoattractants and move preferentially towards those electron acceptors that generate more energy. The mechanism predicts that when electron transport does not occur for a given electron acceptor, taxis to it should also be blocked. The protometer concept is consistent with all electron acceptor taxes reported for *E. coli* and, although the sensor has not been identified, provides a good working framework for understanding *E. coli* taxis.

The results presented here suggest that the situation for *S. putrefaciens* may be very different. The competition studies in which DMSO blocks chemotaxis to nitrate and nitrite (even though respiration of these substrates is normal) suggest that simple utilization of a given electron acceptor is not sufficient for taxis to occur to that compound. The results suggest that DMSO interferes with a sensory system rather than electron flow and are thus inconsistent with the protometer mechanism. In addition, studies with several mutants suggest that in *S. putrefaciens*, taxis does not have an obligate requirement for electron transport and establishment of the proton motive

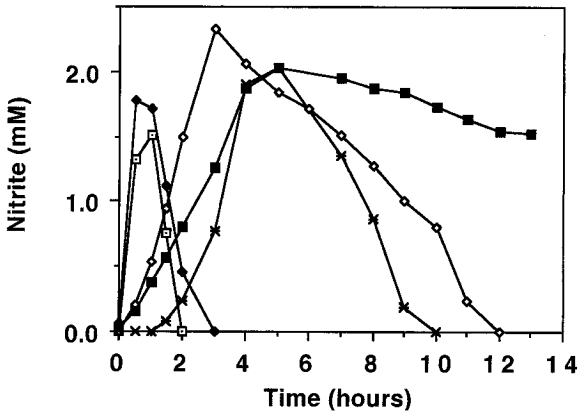

FIG. 2. Nitrite production from nitrate and its consumption by wild-type MR-1 and respiratory mutants (Table 2). Only the *etrA* mutant (MetrA) showed wild-type levels of reduction, with other mutants displaying widely varying respiratory dynamics. No correlation was seen between the rates of electron acceptor utilization and either the degree of taxis towards nitrate or nitrite or the ability of nitrate or nitrite to inhibit taxis of other electron acceptors (Table 2). ⊡, MR-1; ◆, MetrA; ×, MRN36; ◇, M56A; ■, M56.

force by a given electron acceptor. Two mutants that are virtually incapable of nitrate or nitrite reduction (M56 and M56A) show normal taxis to both of these compounds (Fig. 2). Furthermore, Table 2 shows that in some of the mutants, while the reduction of nitrite is perturbed to various degrees (M56 was effectively negative for nitrite reduction), this compound still blocks taxis to fumarate. These results are more consistent with the existence of a receptor and substrate-specific signaling mechanism than with a protometer-like mechanism.

MR-1 showed no chemotactic response to any carbon source tested under aerobic conditions and anaerobically was tactic to only formate (data not shown; 20). Formate chemotaxis is known for strains of *Campylobacter concisus* and *Wollinella succinogenes* (22); *C. concisus* is tactically attracted solely to formate also under anaerobic conditions with fumarate as the electron acceptor.

S. putrefaciens is a nonfermentative organism that dies rapidly after electron acceptor deprivation (16) and is abundant in anoxic environments, especially at redox interfaces (7, 18). Given such metabolic abilities (and limitations), it is not unreasonable to expect that such an organism's tactic abilities might reflect a related metabolic need for electron acceptors. In such nutrient-rich environments, the primary needs may not be a carbon or energy supply but rather the supply of oxidants for respiration. If sensing and positioning relative to electron acceptors are important for survival, it may not be unexpected that anaerobic chemotaxis to electron acceptors in *S. putrefaciens* appears to be different from that proposed for other species of bacteria and is not consistent with the protometer-type mechanism known for the enteric bacteria. Several lines of evidence presented here are consistent with the existence of electron acceptor sensors that depend on the presence rather than the metabolism of these substrates, sensors perhaps similar to those utilized for carbon taxis in other bacteria. Given the chemotactic properties described here, it seems certain that mechanistic investigations of anaerobic chemotaxis of *S. putrefaciens* will reveal some novel adaptations to anaerobic life in stratified environments.

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