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Fe(II) Oxidation Is an Innate Capability of Nitrate-Reducing Bacteria That Involves Abiotic and Biotic Reactions

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Phylogenetically diverse species of bacteria can catalyze the oxidation of ferrous iron [Fe(II)] coupled to nitrate (NO_3^-) reduction, often referred to as nitrate-dependent iron oxidation (NDFO). Very little is known about the biochemistry of NDFO, and though growth benefits have been observed, mineral encrustations and nitrite accumulation likely limit growth. *Acidovorax ebreus*, like other species in the *Acidovorax* genus, is proficient at catalyzing NDFO. Our results suggest that the induction of specific Fe(II) oxidoreductase proteins is not required for NDFO. No upregulated periplasmic or outer membrane redox-active proteins, like those involved in Fe(II) oxidation by acidophilic iron oxidizers or anaerobic photoferrotrophs, were observed in proteomic experiments. We demonstrate that while "abiotic" extracellular reactions between Fe(II) and biogenic NO_2^-/NO can be involved in NDFO, intracellular reactions between Fe(II) and periplasmic components are essential to initiate extensive NDFO. We present evidence that an organic cosubstrate inhibits NDFO, likely by keeping periplasmic enzymes in their reduced state, stimulating metal efflux pumping, or both, and that growth during NDFO relies on the capacity of a nitrate-reducing bacterium to overcome the toxicity of Fe(II) and reactive nitrogen species. On the basis of our data and evidence in the literature, we postulate that all respiratory nitrate-reducing bacteria are innately capable of catalyzing NDFO. Our findings have implications for a mechanistic understanding of NDFO, the biogeochemical controls on anaerobic Fe(II) oxidation, and the production of NO_2^-, NO, and N_2O in the environment.

Although microbial nitrate-dependent Fe(II) oxidation (NDFO) has been known for over 2 decades and has been demonstrated to play a critical role in both the extant (1) and ancient (2) global iron cycles, almost nothing is known of the underlying biochemical or genetic mechanisms involved (3). NDFO microbes have been demonstrated to oxidize both soluble and insoluble Fe(II) (1, 4–6) and to produce a variety of insoluble mixed-valence iron mineral products (1, 5, 7, 8).

Some evidence already exists that the capacity for NDFO is widespread and likely innate to all nitrate-reducing bacteria. Bacterial species that couple the oxidation of Fe(II) to nitrate reduction have been isolated from a wide range of habitats and are phylogenetically diverse, indicating their environmental prevalence and importance (5, 9, 10). Recently, species of the genus *Acidovorax* have dominated enrichment cultures and been isolated as proficient nitrate-dependent Fe(II) oxidizers (11–14); however, even *Escherichia coli* has been demonstrated to be capable of Fe(II) oxidation coupled to nitrate reduction (15). Although NDFO is based on thermodynamically favorable redox reactions (16, 17), only a few studies have suggested growth enhancement from this metabolism (12, 18). This suggests that NDFO may be primarily inadvertent or a detoxification strategy.

While the toxicity of Fe(II) to biological systems under aerobic conditions is widely appreciated, less is understood about Fe(II) toxicity under anaerobic conditions. Some studies have demonstrated that Fe(II) at low concentrations is toxic to anaerobic bacteria such as anoxygenic phototrophs (19) or streptococci (20). Several possibilities for anaerobic Fe(II) toxicity include inhibition of the F-ATPase (20), binding to membranes (21), disruption of protein stability or replacement of active-site metal cofactors (22), and oxidation and precipitation of insoluble Fe(III) on cellular components to impair nutrient uptake (13). Under NDFO conditions, redox transformations of nitrogen oxides can produce intermediates, such as nitric oxide (NO), which is capable of binding to and reacting with heme cofactors (23) or Fe-S clusters (24) or, in the presence of transition metals, nitrosating protein thiols to inhibit or alter protein activity (25).

Here we present the results of proteomic and physiological experiments used to understand the mechanism of NDFO in the model organism *Acidovorax ebreus*. Like several other species in the *Acidovorax* genus (12–14), *A. ebreus* was isolated on the basis of its capacity for NDFO (11). Previous studies have reported a growth advantage from NDFO by *Acidovorax* species in both batch and continuous-flow cultures (12, 14), in addition to the accumulation of nitrite (NO_2^-) and periplasmic mineral encrustations (26). On the basis of our findings with *A. ebreus*, we postulate that all nitrate-respiring organisms are innately capable of catalyzing NDFO, but the survival and growth benefits obtained are dependent on the ability to overcome Fe(II), NO_2^-, and NO toxicity. Our findings have implications for understanding the evolution of Fe(II) oxidation as a microbial metabolism, the impact of Fe(II) on nitrate-reducing communities, and the influence of Fe(II) on the product distribution of microbial nitrate reduction.

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MATERIALS AND METHODS

Media and culture conditions. Anaerobic cultures of *A. ebreus* strain TPSY, formerly *Diaphorobacter* sp. strain TPSY (11), and *Azospira suillum* strain PS were grown organotrophically in anoxic bicarbonate-buffered basal medium (BBM) (27), pH 6.8, at 37°C with 10 mM sodium nitrate and various concentrations of sodium acetate and harvested in late log phase. BBM contained (per liter) 0.25 g NH_4Cl, 0.6 g NaH_2PO_4, 0.1 g KCl, and 2.52 g $NaHCO_3$ with the addition of vitamins and minerals according to Bruce et al. (27) and the appropriate concentration of electron donors and acceptors.

Addition of millimolar Fe(II) to the growth medium caused the immediate formation of a white precipitate leaving 1 to 2 mM soluble Fe(II). This precipitate was shown to be predominantly vivianite by Kappler et al. (13). Unless otherwise indicated, our growth and cell suspension experiments utilized this mixed soluble- and insoluble (vivianite)-Fe(II) form.

Fe(II) oxidation cell suspensions. Anaerobic cultures of *A. ebreus* were grown organotrophically in BBM with 10 mM sodium nitrate and 6.25 mM sodium acetate and harvested in late log phase. Cells were washed three times in BBM without donors or acceptors under an N_2-CO_2 (80:20%, vol/vol) headspace. In cell suspensions, cells were inoculated into fresh medium at ~5×10^8/ml with the electron donor and acceptor concentrations indicated. When indicated, chloramphenicol was added to cell suspensions at a concentration of 100 μg/ml. Chloramphenicol was found to completely inhibit the growth of *A. ebreus* at concentrations above 50 μg/ml. When indicated, Fe(II) in the form of Fe(II)-nitrilotriacetic acid (NTA), Fe(II)-NTA-agarose, or synthetic vivianite was added from anoxic stock solutions. Fe(II)-NTA-agarose was prepared by adding $FeCl_2$ from sterile anoxic stock solutions to NTA-agarose beads (Qiagen). The beads were washed five times with sterile water under anoxic conditions. Synthetic vivianite was made by chemical precipitation. $FeCl_2$ was added to 10 mM phosphate buffer. The precipitate was washed with 10 mM phosphate buffer and kept as a suspension under anoxic conditions. To assess the capacities of NO and NO_2^- to oxidize and solubilize Fe(II) from synthetic vivianite, anoxic aqueous stock solutions of sodium nitrite or diethylamine (DEA) NONOate (Cayman Chemical) in 0.1 M NaOH were added to BBM containing synthetic vivianite. NONOates, or diazeniumdiolates, are stable at alkaline pH but decompose at neutral pH to release NO (28). DEA NONOate decays at pH 7.4 and 25°C with a 16-min half-life to release 2 mol of NO/mol of the parent compound. Appropriate controls for the effect of DEA (the product of NONOate decay) and sodium hydroxide were used to show that neither of these compounds, at the concentrations added to generate NO, altered the oxidation state or solubility of Fe(II) in synthetic vivianite.

Analytical techniques. Nitrate, nitrite, and acetate were measured with an ion chromatograph (ICS-2100; Dionex, Sunnyvale, CA) by a method developed to quantify all three analytes simultaneously. Samples for ion chromatography were diluted in 10 mM NaOH, filtered, and stored at −20°C until analysis. The guard and analytical columns were IonPac AG16 (4 by 50 mm) and IonPac AS16 (4 by 250 mm), respectively, with an ASRS-300 4-mm suppressor system and a DS6 heated conductivity cell. A KOH gradient was generated with the EGC III KOH generator at an isocratic flow rate of 1.5 ml/min. The KOH concentration was 1.5 mM from 0 to 7 min, ramped to 10 mM from 7 to 13 min, held at 10 mM from 13 to 16 min, ramped to 35 mM from 16 to 17 min, held at 35 mM from 17 to 27 min, and ramped back down to 1.5 mM from 27 to 30 min.

The soluble and insoluble forms of Fe(II) were measured separately to characterize the oxidation of each Fe(II) form in cultures and to avoid analytical artifacts due to rapid reactions that occur between NO_2^- and Fe(II) in acidic solution and the regeneration of NO_2^- through reactions between NO and O_2 under oxic conditions. Klueglein and Kappler (29) have provided an excellent discussion of this problem and other alternatives to avoid it. At each time point, 0.5 ml of culture medium was removed in the anaerobic chamber (Coy) and centrifuged to separate insoluble Fe(II) from soluble Fe(II) and NO_2^- in solution. Soluble Fe(II) was directly measured with the ferrozine assay, and insoluble Fe(II) was resuspended in 0.5 M HCl for 24 h and Fe(II) was quantified by the ferrozine assay (30). Control experiments in which insoluble Fe(II) was washed with anoxic buffer to remove residual nitrite prior to resuspension in 0.5 M HCl gave identical ferrozine assay results.

Two techniques were used to measure NO gas. For headspace NO analysis, culture headspace was anaerobically sampled and injected into the purge cell of a 280i NO analyzer (Sievers). For solution NO, samples of culture solution were injected into a purge solution in anoxic vials (31). The headspace from these vials was injected into the NO analyzer. The purge solution releases NO from iron complexes in solution and contains 5.5 ml glacial acetic acid, 1.5 ml 0.8 M potassium ferricyanide, and 1 ml 0.1 M sulfanilamide in 2 M glacial acetic acid. Ferricyanide oxidizes iron and releases NO, and sulfanilamide reacts rapidly with NO_2^- to prevent further NO release in the acid solution. NO gas standards were used to generate a standard curve for quantification.

For measurement of N_2O, culture headspace (2 ml) was collected with a gas-tight syringe and injected directly into a Hewlett Packard HP6890 (Agilent Technologies Inc., Santa Clara, CA) gas chromatograph fitted with a Hayesep DB 100/120 column (1/16 in. by 1.5 m) that fed into a Hayesep DB 120/140 column (1/16 in. by 2.0 m) leading to a pulse discharge detector (PDD). The PDD was calibrated for N_2O by using a five-point standard curve, and a single standard was analyzed hourly thereafter to correct for instrumental drift.

Growth in cultures was monitored by direct counts of acridine orange-stained cells. At various time points, 500-μl culture volumes were fixed in 3.7% formaldehyde. Cells were then diluted in water or 100 mM oxalate buffer. One milliliter of diluted cells was stained with acridine orange (final concentration, 1 μg/ml) and applied to a polycarbonate black filter in a glass vacuum filter apparatus (Fisher). The filter was washed with 5 ml of oxalate buffer, and cells were counted by microscopic inspection with an oil immersion lens at ×100 magnification in an Axioimager M1 microscope (Zeiss).

Trypsin-shaving proteomic sample preparation. For proteomics, cells from NDFO and organoheterotrophic cultures were harvested anaerobically at time points between 0 and 48 h. At each time point, 50 ml of culture was centrifuged anaerobically, treated with oxalate buffer to remove Fe(II) from the cells, and washed twice with 100 mM ammonium bicarbonate buffer. Cells were resuspended in 2 aliquots of 500 μl. One sample was lysed (for a whole-cell lysate proteome), and the other was kept intact (for a trypsin-shaved proteome) under anaerobic conditions. Two hundred nanograms of Trypsin Gold porcine protease (Pierce) was added to lysed and intact cells, and samples were incubated for 45 min at 37°C. After 45 min, both the lysed and intact cells were centrifuged at a relative centrifugal force of 10,000 and the supernatant was treated in two sequential 30-min steps with 1 mM dithiothreitol and 1 mM iodoacetamide and then further digested overnight with 200 ng of trypsin at 37°C. Peptides from overnight digests were concentrated with 100-μl C_{18} OMIX tips (Agilent) and eluted with 85% acetonitrile–0.1% trifluoroacetic acid in water. Acetonitrile was removed by vacuum centrifugation (SpeedVac), and peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (see the supplemental material).

Proteomic data analysis. Normalized peptide counts were used as a semiquantitative measurement of relative protein abundance. Validation of this approach has been previously published (32, 33). Peptide counts were normalized by dividing the number of peptides observed for a given protein by the total number of peptides observed in a sample. For statistical analysis, normalized peptide counts in intact and lysed samples were pooled for a given growth condition by combining the data from all of the time points across the growth curve. Student's *t* test was used to compare the normalized peptide counts from NDFO and organotrophic growth conditions. *P* values of ≤0.05 were considered to be significant.

RESULTS AND DISCUSSION

Evidence against an inducible Fe(II) oxidoreductase in *A. ebreus*. Previous studies of anaerobic Fe(II) oxidation by pho-

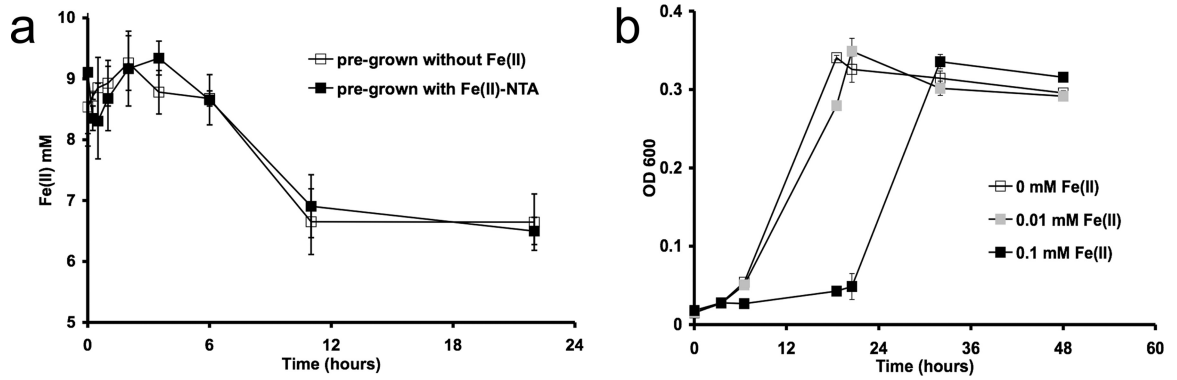

FIG 1 NDFO of mixed soluble and insoluble Fe(II) is not inducible, but Fe(II) is toxic to nitrate-reducing *A. ebreus*. (a) Concentration of Fe(II) (soluble and vivianite) in chloramphenicol-treated washed cell suspensions of *A. ebreus*. Cells were pregrown organotrophically (10 mM acetate, 10 mM nitrate) in the absence of Fe(II) or under NDFO conditions in the presence of Fe(II)-NTA [10 mM Fe(II)-NTA, 5 mM acetate, 10 mM nitrate]. (b) Growth expressed as optical density at 600 nm (OD 600) in organotrophic cultures (5 mM acetate, 10 mM nitrate) of *A. ebreus* containing 0, 0.01, or 0.1 mM Fe(II). Points represent averages of triplicates, and error bars represent standard deviations.

totrophic organisms demonstrated induction of putative Fe(II) oxidoreductase proteins and enhanced Fe(II) oxidation when cells were precultured in the presence of Fe(II)-NTA (34). In contrast, preculturing of *A. ebreus* on Fe(II)-NTA was not required for NDFO of mixed insoluble (vivianite/siderite) and soluble Fe(II) in chloramphenicol-treated washed cell suspensions without acetate, indicating a basal capacity for NDFO (Fig. 1a). Similar results were obtained in the absence of chloramphenicol (see Fig. S1 in the supplemental material). Furthermore, preculturing in the presence of Fe(II)-NTA did not enhance the rate or extent of Fe(II) oxidation of mixed soluble and insoluble Fe(II) (Fig. 1a).

In the model acidophilic Fe(II) oxidizer *Acidithiobacillus ferrooxidans*, outer membrane multiheme *c*-type cytochromes and rusticyanin, a periplasmic copper protein, are proposed to be involved in Fe(II) oxidation (35). In the phototrophic iron oxidizers *Rhodobacter* sp. strain SW2 and *Rhodopseudomonas palustris* TIE-1, outer membrane porins (PioB), periplasmic multiheme *c*-type cytochromes (PioA/FoxE), and periplasmic high-potential iron proteins (PioC) or quinoproteins (FoxY) are involved (35). Similar *c*-type cytochrome-based mechanisms are proposed for microaerophilic Fe(II) oxidation (36). While a number of *c*-type cytochrome genes are present in the *A. ebreus* genome, there are no multiheme *c*-type cytochromes or close homologs of the *pio*/*fox* genes (35). The Dtpsy_1207, Dtpsy_1208, and Dtpsy_2198 gene products have homology with multicopper oxidase family proteins, some of which are known to be involved in Mn(II) oxidation, but these proteins typically utilize oxygen as a cosubstrate to oxidize metals (37) and were never observed in proteomic experiments; therefore, they are unlikely to be involved in NDFO by *A. ebreus*.

To search for an inducible Fe(II) oxidoreductase, we compared the whole-cell lysate and trypsin-shaved proteomes of organotrophic (5 mM acetate, 10 mM nitrate) and NDFO [10 mM Fe(II), 5 mM acetate, 10 mM nitrate] growth cultures of *A. ebreus* at 8, 18, 24, 32, and 48 h. Representative growth curves and data on the transformation of Fe(II), acetate, and nitrogen oxides are presented in Fig. S2 in the supplemental material. Trypsin shaving has previously been shown to be useful for identifying surface-exposed proteins, including cytochromes, in other bacteria (38, 39), and we used it to increase our chance of identifying outer membrane proteins that may be contacting Fe(II) and catalyzing cell surface Fe(II) oxidation. When the proteomes of cells harvested at time points between 8 and 48 h from NDFO and organotrophic *A. ebreus* growth cultures are compared, significantly increased abundance ($P \leq 0.05$) is observed for heavy metal RND (resistance-nodulation-division) family efflux pumps Dtpsy_1460 ($P = 0.018$), Dtpsy_1461 ($P = 0.0030$), and Dtpsy_1462 ($P = 0.00070$) (Fig. 2; see Data Set S1 in the supplemental material). In the set of extracytoplasmic proteins that were observed exclusively in NDFO cultures (see Data Set S1E), only a few peptides were observed, but efflux pumps are highly represented. The expression of heavy metal efflux pumps in response to anaerobic Fe(II) stress is a novel finding and suggests that these proteins may be involved in the export of metals other than those commonly considered to be substrates (40, 41). Furthermore, because the RND family efflux pumps identified in *A. ebreus* are proton/solute antiporters, the energy cost is putatively less than that of an ATP-consuming transporter and RND family efflux pumps are able to load substrate from either the periplasm or the cytoplasm (42).

The proteomic data also suggest a cytoplasmic response to redox/nitrosative stress. Although the *P* values are higher (see Data Set S1 in the supplemental material), dihydrolipoamide dehydrogenase subunits of the pyruvate dehydrogenase complex, Dtpsy_1140 ($P = 0.074$) and Dtpsy_1658 ($P = 0.070$), which are known to be sensitive to NO (43), were more abundant. Also more abundant were proteins involved in fatty acid synthesis, enoyl coenzyme A hydratase, Dtpsy_2933 ($P = 0.11$), and a member of the 2-nitropropane dioxygenase family, Dtpsy_2296 ($P = 0.055$), that is closely related to a *Neisseria gonorrhoeae* homolog (NGO1024) putatively involved in anaerobic, NO-dependent fatty acid synthesis (44). Aconitase, Dtpsy_1066 ($P = 0.25$), which is sensitive to nitrosative stress (45), and the large subunit of NO reductase, Dtpsy_0109 ($P = 0.13$) (46), were also observed more often in Fe(II) cultures, but the *P* values are higher (see Data Set S1). Two proteins, Dtpsy_0452 ($P = 0.030$) and Dtpsy_1098 ($P = 0.045$), were significantly less abundant in NDFO cultures of *A. ebreus* (see Data Set S1). Given that peptides from core periplasmic and inner membrane respiratory proteins were observed in the proteomic experiments, it is unlikely that a respiratory ferroxidase was missed. Several periplasmic *c*-type cytochromes were ob-

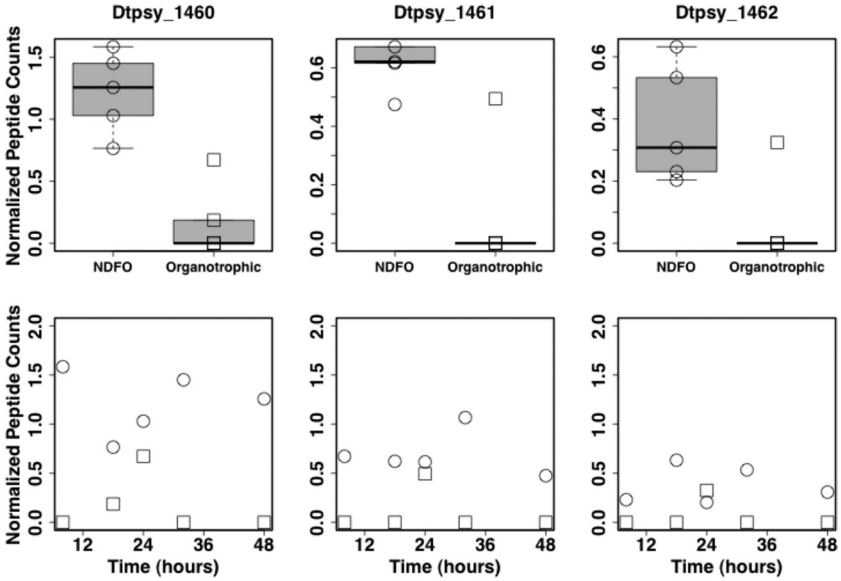

FIG 2 Heavy metal efflux pumps are more abundant in NDFO cultures. Normalized peptide counts of Dtpsy_1460, Dtpsy_1461, and Dtpsy_1462 in NDFO and organotrophic cultures at 8, 18, 24, 36, and 48 h. (Top row) Box plots showing the lower extreme, lower quartile, median, upper quartile, and upper extreme of normalized peptide counts. Data from the time courses were pooled from trypsin-shaving proteomic experiments. Data are overlaid on the box plots. (Bottom row) Normalized peptide count data at 8, 18, 24, 36, and 48 h in NDFO and organotrophic cultures.

served (see Data Set S1), including Dtpsy_0754, a component of the cytochrome bc_1 complex, but there was no significant difference in their abundance between organotrophic and NDFO proteomes (see Data Set S1). Overall, the proteomic response to Fe(II) is consistent with a cell envelope and cytoplasmic stress response to Fe(II) and nitrogen oxides rather than the induction of respiratory metabolism.

Fe(II) is toxic to nitrate-reducing cultures of *A. ebreus*. In support of Fe(II) toxicity to nitrate-reducing cells, when 0.1 mM Fe(II) was added to organotrophic cultures of *A. ebreus*, a growth lag of 12 to 18 h was observed (Fig. 1b). Similar concentrations of Fe(II) are inhibitory to the growth of *Rhodobacter capsulatus* grown under humic acid-oxidizing phototrophic conditions (19). A lag was also observed in NDFO cultures of *A. ebreus* containing higher Fe(II) concentrations (8 to 10 mM) (see Fig. S2b and c in the supplemental material), and NO_2^- and NO both accumulated to at least 10-fold higher levels in the NDFO cultures than in organotrophic (no Fe) cultures (see Fig. S2d and e). The growth lag in NDFO cultures was partially relieved by the addition of NTA, with an accompanying increase in the rate of Fe(II) oxidation (see Fig. S3a and b). We also observed less NO accumulation in the headspace of NDFO cultures grown with Fe(II)-NTA than in that of cultures grown with unchelated Fe(II) (see Fig. S3c). NO toxicity is likely less of a problem in the presence of Fe(II)-NTA, which forms a stable NO complex with a submicromolar K_d (dissociation constant) (47).

Possible mechanisms of NDFO by *A. ebreus* and other nitrate-reducing bacteria. In the absence of an inducible Fe(II) oxidoreductase, one mechanism or a combination of several mechanisms of NDFO are possible, including (i) direct electron donation from Fe(II) to respiratory complexes if the appropriate electron acceptor is available (i.e., NO_3^- for Nar, NO_2^- for Nir, etc.), (ii) extracellular or intracellular mineral phase Fe(II) oxidation coupled to the reduction of NO_3^- or biogenic NO_2^- and NO (48) (green rusts have been shown to form during NDFO by *Acidovorax* sp. strain BoFeN1 [49]), (iii) proton-dependent abiotic Fe(II) reduction of NO_2^- under the lower-pH conditions that exist in the bacterial periplasm, and (iv) Fe(II) oxidation coupled to the reduction of NO_2^- and NO catalyzed by periplasmic components such as protein thiols (3, 41, 50) or membrane-bound iron (51, 52).

Fe(II) must enter the periplasm for extensive oxidation by *A. ebreus*. NDFO catalysis in the periplasm requires that Fe(II) cross the outer membrane, which, though porous, is negatively charged and binds Fe(II) (52). A long lag (~6 h) precedes Fe(II) oxidation (Fig. 1a) and nitrate reduction (see Fig. S5a in the supplemental material) in cell suspensions with mixed soluble and insoluble Fe(II), consistent with the outer membrane acting as a barrier to Fe(II) entry into the periplasm (Fig. 1a; see Fig. S1 and S5a). When *A. ebreus* cells are lysed, releasing periplasmic components, NDFO commences earlier (Fig. 3a). Fe(II)-NTA, which does not adsorb to cell surfaces because the Fe(II) is bound by a chelator, is oxidized rapidly in the first 6 h of incubation (Fig. 3b). A more extensive treatment of the accelerating effect that metal ligands have on the rate of microbial Fe(II) oxidation was recently published (53), and the authors demonstrated that NO_2^- reacts more rapidly with Fe(II)-NTA than with unchelated Fe(II), suggesting another possible explanation for the high rate of Fe(II)-NTA oxidation by *A. ebreus* (Fig. 3b) (53). However, Fe(II)-NTA-agarose beads, which cannot penetrate the outer membrane, showed no oxidation by *A. ebreus* cell suspensions (Fig. 3b). This result suggests that Fe(II)-NTA must enter the periplasm to be oxidized and argues against the involvement of inducible outer membrane redox-active proteins or a secreted oxidant in NDFO of Fe(II)-NTA by *A. ebreus*. Previous observations from a scanning transmission X-ray microscopy study found that the rate of Fe(II) oxidation by *Acidovorax* BoFeN1 is faster at the cells, and specifically in the periplasm, than at the extracellular Fe(II) (26). This is consistent

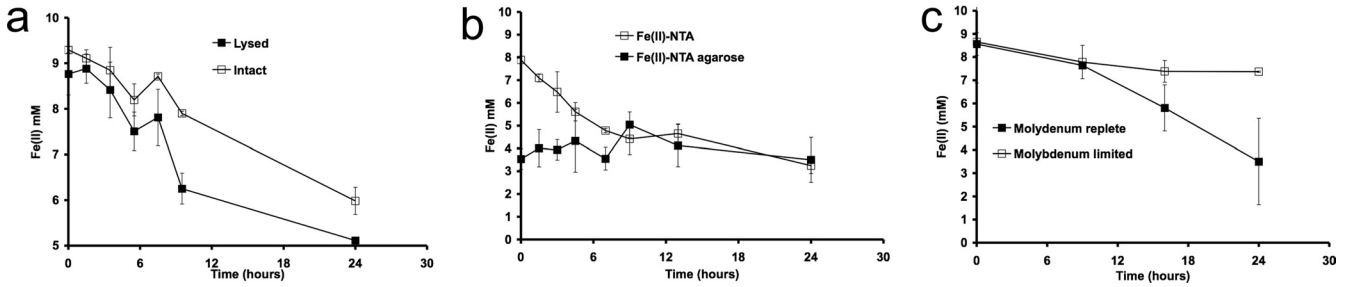

FIG 3 Periplasmic reactions are essential to initiate extensive NDFO. (a) Concentration of Fe(II) in NDFO cell suspensions of lysed or intact *A. ebreus* in the presence of chloramphenicol in medium containing 10 mM Fe(II) and 10 mM nitrate. (b) Concentration of Fe(II) as Fe(II)-NTA or Fe(II)-NTA agarose beads in NDFO cell suspensions [10 mM Fe(II), 5 mM acetate, 10 mM nitrate] in the absence of chloramphenicol. (c) Concentration of Fe(II) in NDFO cell suspensions of molybdenum-depleted *A. ebreus* in the presence of chloramphenicol in either molybdenum-free or molybdenum-replete medium [10 mM Fe(II), 10 mM nitrate]. Cells of *A. ebreus* were pregrown in molybdenum-free medium (5 mM acetate, 10 mM nitrite, 10 mM nitrate) and were incapable of growth by organotrophic nitrate reduction (not shown). Points represent averages of triplicates, and error bars represent standard deviations.

with the idea that periplasmic access of Fe(II) is required for robust NDFO by members of the *Acidovorax* genus.

We sought to understand how NDFO occurs in the periplasm. Cytochrome complexes of intact cells are reduced by Fe(II)-NTA (see Fig. S4 in the supplemental material), as has previously been observed with other organisms (5, 18), and acetate is not required for NDFO in cell suspensions (Fig. 1a). Thus, we hypothesized that respiratory complexes could directly mediate NDFO. To test this, we deprived cells of molybdenum, the metal cofactor of the nitrate reductase Nar. *A. ebreus* cells grown anaerobically in the absence of molybdenum under nitrite-reducing conditions, but with NO_3^- available to induce Nar expression, were unable to grow by organotrophic NO_3^- reduction (data not shown) and incapable of Fe(II) oxidation coupled to NO_3^- reduction in cell suspensions (Fig. 3c). These results suggest that Nar directly catalyzes NO_3^- reduction coupled to Fe(II) oxidation (54) and/or NO_2^- is produced to further react with Fe(II) through the abiotic and biotic mechanisms previously discussed.

Both nitrite and NO can oxidize extracellular solid-phase Fe(II). Previously, it was observed that synthetic solid-phase iron phosphate (vivianite) in the absence of soluble Fe(II) is not oxidized by growth cultures of *Acidovorax* species (13, 26). In contrast, we found that small amounts of synthetic vivianite [~0.5 mM Fe(II) over the course of 24 h] can be oxidized by cell suspensions of *A. ebreus* but only in the presence of an organic cosubstrate (Fig. 4a). As the cell suspensions were treated with chloramphenicol, it is unlikely that the cells produced an organic chelator as a result of acetate amendment. An alternative option is that intermediates of nitrate respiration escape the periplasm and solubilize and/or oxidize solid-phase iron. Candidates for a secreted oxidant include NO_2^- and, when soluble Fe(II) is also present in the system, Fe(III) (13, 26). Some NDFO organisms, such as *Pseudogulbenkiania ferrooxidans* (67) or *Pseudogulbenkiania* sp. strain MAI-1 (53), release higher concentrations of extracellular NO_2^- during organotrophic nitrate reduction, which can react with extracellular Fe(II), especially in the presence of catalytic mineral phases, such as green rusts (21, 48, 49), or chelating ligands (53). However, NO should also be considered. NO is produced during organotrophic nitrate reduction by *A. ebreus* (see Fig. S2e in the supplemental material) and other nitrate reducers (55, 56). NO oxidizes Fe(II) and is a good Fe(II) ligand that displaces a number of anionic ligands (57). NO oxidizes synthetic vivianite Fe(II) faster than NO_2^- does (Fig. 4c). After 15 min, ~0.5 mM synthetic vivianite Fe(II) was oxidized by 0.5 mM DEA NONOate but no noticeable synthetic vivianite Fe(II) oxidation occurred in the presence of 1 mM NO_2^- (Fig. 4c). Even low steady-state concentrations of NO may be enough to account for the small amount of synthetic vivianite oxidized when acetate is added to dense cell suspensions (Fig. 4a). NO can also solubilize Fe(II) from synthetic vivianite (Fig. 4b) and increase the rate of Fe(II) oxidation via the

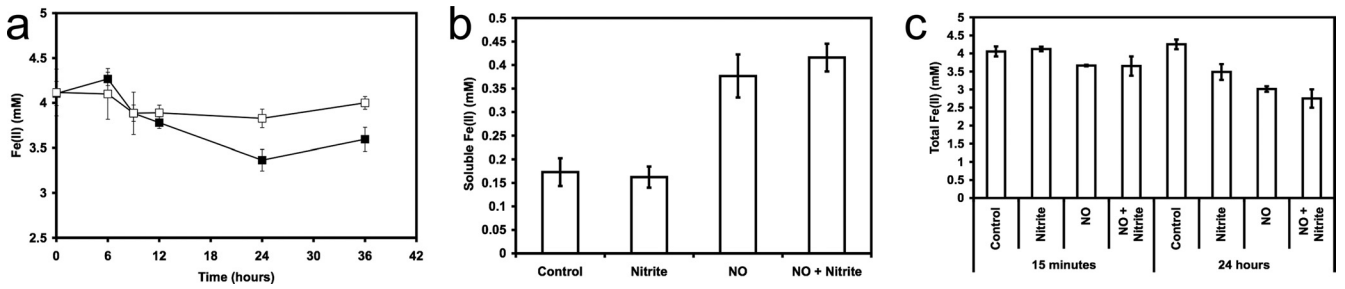

FIG 4 Extracellular reactions between NO_2^- and NO and mineral phase Fe(II) can contribute to NDFO. (a) Concentration of Fe(II) with synthetic vivianite as the sole Fe(II) source in NDFO cell suspensions of *A. ebreus* [4 mM Fe(II), 10 mM nitrate, and 0 or 5 mM acetate] in the presence of chloramphenicol. (b) Concentration of soluble Fe(II) after 15 min in cell-free basal medium containing 4 mM Fe(II) in the form of synthetic vivianite and either 1 mM sodium nitrite (NO_2^-), 0.5 mM DEA NONOate (NO), or both compounds. Controls contained DEA and sodium hydroxide and showed no Fe(II) oxidation over the time course of the experiment. (c) Total Fe(II) concentration after 15 min and 24 h in cell-free basal medium containing 4 mM Fe(II) in the form of synthetic vivianite and either 1 mM sodium nitrite, 0.5 mM DEA NONOate (NO releasing compound), or both compounds. Controls contained DEA and sodium hydroxide and showed no Fe(II) oxidation over the time course of the experiment. Points represent averages of triplicates, and error bars represent standard deviations.

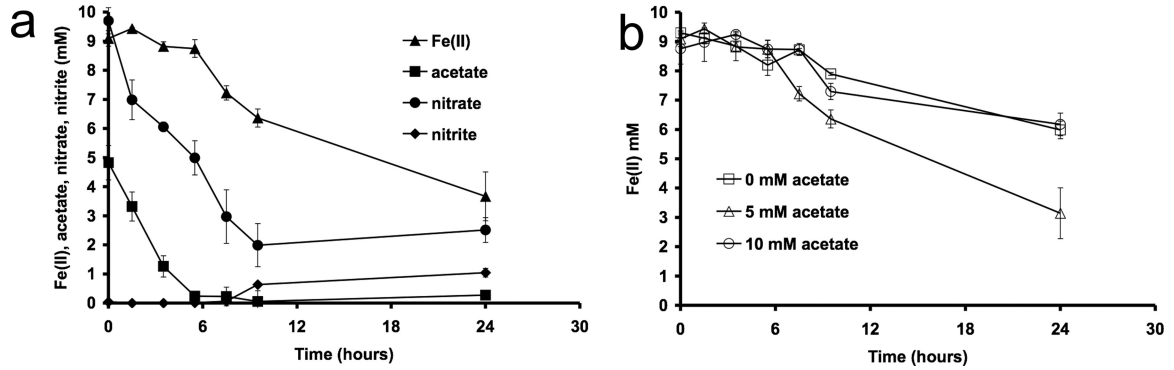

FIG 5 Acetate is consumed prior to Fe(II) as an electron donor in nitrate-reducing cultures. (a) Concentrations of Fe(II), acetate, nitrate, and nitrite in NDFO cell suspensions of *A. ebreus* [10 mM Fe(II), 5 mM acetate, 10 mM nitrate] in the presence of chloramphenicol. Points represent averages of triplicates, and error bars represent standard deviations. (b) Fe(II) concentrations in NDFO cell suspensions of *A. ebreus* with 10 mM Fe(II); 10 mM nitrate; and 0, 5, or 10 mM acetate. Points represent averages of triplicates, and error bars represent standard deviations.

periplasmic reactions previously discussed. We saw accumulation of solution NO (see Fig. S6a) in later phases of cultures along with a rebound in the concentration of soluble Fe(II) (see Fig. S6b). The observation that NO and, to a lesser extent, NO_2^- can oxidize solid-phase extracellular Fe(II) (Fig. 4c) is ostensibly at odds with the observation that Fe(II)-NTA agarose was not oxidized by *A. ebreus* (Fig. 3b). However, NO binds to Fe(II)-NTA with nanomolar affinity to form stable Fe(II)-NTA-NO complexes (58) but does not oxidize chelated Fe(II) (47). Although abiotic oxidation of Fe(II)-NTA by NO_2^- is faster than that of the unchelated form at neutral pH (53), negligible amounts of NO_2^- are produced by *A. ebreus* during organotrophic denitrification (see Fig. S2d).

It has been proposed that a low local pH around iron-oxidizing cells could keep Fe(III) soluble and allow it to diffuse out of cells to react with extracellular vivianite (26). However, we have direct evidence of NDFO-associated efflux pumps from proteomics (Fig. 2; see Data Set S1), suggesting a possible active mechanism of Fe(III) secretion from cells to react with extracellular Fe(II) minerals through an electron exchange mechanism. RND family efflux pumps have relaxed specificity and can transport a wide range of metal ions (40). This electron exchange mechanism may involve reactions between extracellular colloidal iron and extracellular minerals (13, 26). Previous reports that found NDFO of solid-phase Fe(II) in batch cultures could be explained by a combination of the solubilization of Fe(II) by NO; oxidation by NO, NO_2^-, and Fe(III); and lysis and cryptic growth during long incubation periods (4, 5, 59).

Acetate is utilized before Fe(II) as an electron donor for nitrate reduction, and excess acetate inhibits NDFO and NO_2^-, NO, and N_2O accumulation. It was previously noted that acetate is consumed before Fe(II) in NDFO growth cultures (5, 60), and this is also true for *A. ebreus* (see Fig. S2b in the supplemental material). For other *Acidovorax* species in growth culture under donor-limited conditions, increasing acetate concentrations increased the extent of iron oxidation (12), likely because, at least in part, of the increasing biomass. However, we observed that under acceptor-limited conditions in growth cultures, excess acetate decreased the rate and extent of Fe(II) oxidation and NO_2^-, NO, and N_2O accumulation by *A. ebreus* (see Fig. S2 and S7a). To understand the effect of acetate on iron oxidation without growth or cell number as confounding variables, we prepared cell suspensions with chloramphenicol. Remarkably, the presence of acetate also controls Fe(II) oxidation and NO_2^- and NO accumulation in cell suspensions (Fig. 5; see Fig. S5). Under balanced donor-acceptor conditions [10 mM Fe(II), 5 mM acetate, 10 mM nitrate], the most rapid phase of Fe(II) oxidation occurred once acetate was depleted to below 0.5 mM (between 24 and 36 h) (Fig. 5a). A similar rapid phase of Fe(II) oxidation at acetate concentrations below 0.5 mM is observed in NDFO growth cultures (see Fig. S2b). However, under acceptor-limited conditions [10 mM Fe(II), 10 mM acetate, 10 mM nitrate], this effect was greatly diminished because acetate was consumed prior to over Fe(II) (Fig. 5b; see Fig. S2c and S5b). Consistent with the observations made with *A. ebreus*, acetate is consumed before Fe(II) in NDFO growth cultures of *A. suillum* strain PS (5) (see Fig. S7b) and excess acetate inhibits Fe(II) oxidation under acceptor-limited conditions (see Fig. S7c in the supplemental material).

A recent paper by Klueglein and Kappler (29) claimed that enzymatic NDFO is unlikely to occur in *Acidovorax* sp. strain BoFeN1, in part on the basis of the fact that the organism does not oxidize an initial concentration of 7 mM Fe(II) in a growth culture with 5 mM acetate (40 mM electron equivalents), 100 μM NO_3^- (0.5 mM electron equivalents), and ~20 mM N_2O (40 mM electron equivalents) (5% added to headspace). If acetate is preferentially consumed by *Acidovorax* sp. strain BoFeN1, very few electron equivalents of N_2O will remain for Fe(II) oxidation to proceed coupled to N_2O reduction. The authors mention, but do not show, the data from a similar growth experiment with 0.5 mM acetate instead of 5 mM acetate. Of course, in this instance, very little growth is expected and the concentration of cells available to catalyze NDFO would be very low. While our results suggest that NDFO, particularly of chelated Fe(II) (Fig. 2b), can be catalyzed in the periplasm of nitrate-reducing bacterial cells, more work is needed to define precisely the catalytic sites.

A model of NDFO as an innate capability of nitrate-reducing bacteria. In NDFO growth cultures, when acetate is present, periplasmic respiratory complexes remain reduced and NO/NO_2^- concentrations remain low. Also, metal efflux pumps may function to keep Fe(II) out of the periplasm (Fig. 2) (61). When acetate is depleted and respiratory complexes are oxidized, Fe(II) efflux is slowed and periplasmic NDFO reactions can proceed. Fe(II) oxidation coupled to reduction of intermediates of nitrate

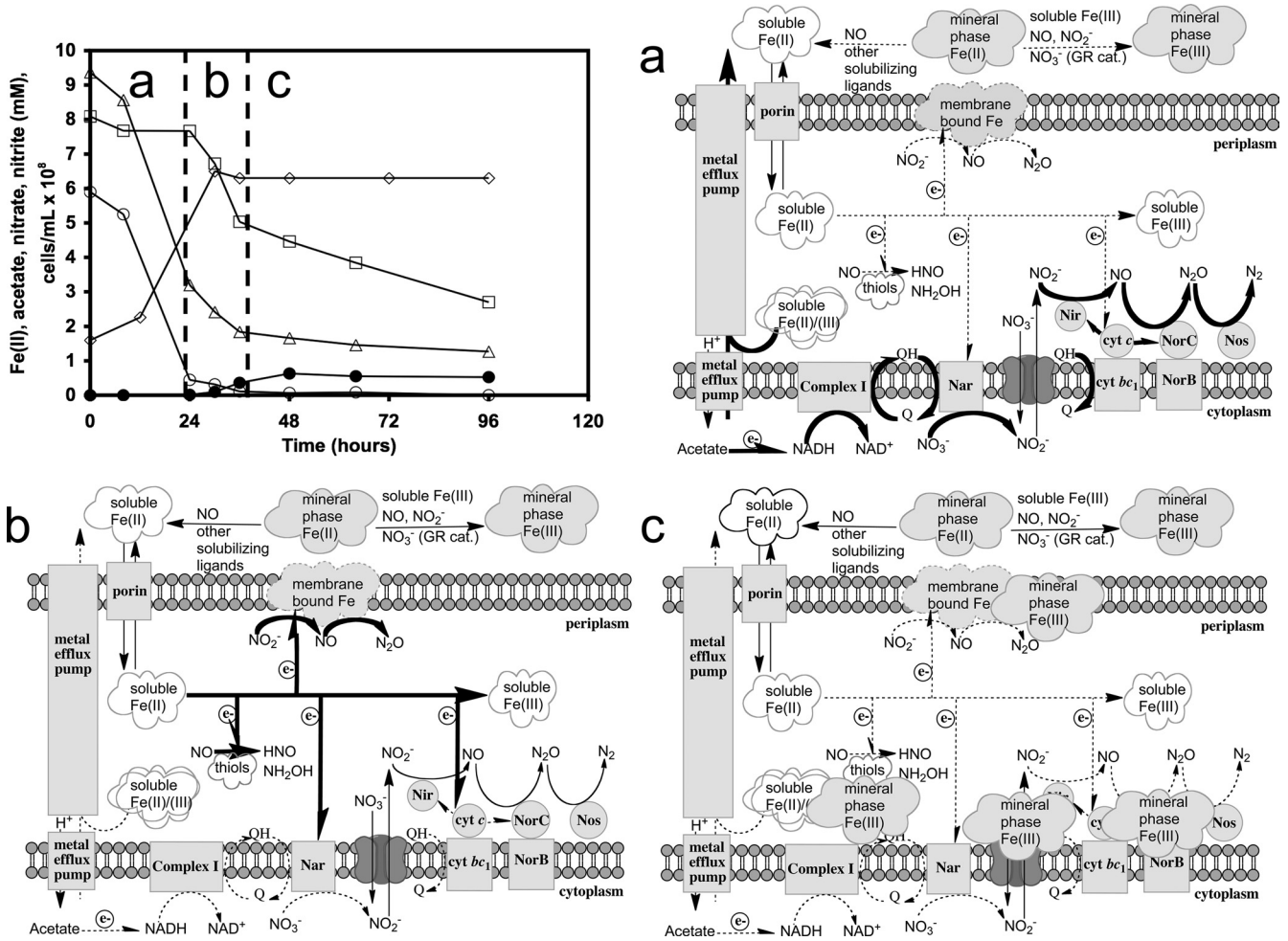

FIG 6 A mechanistic model of nitrate-dependent Fe(II) oxidation by *A. ebreus*. Across a representative NDFO growth curve, dominant electron flow pathways are proposed for before (a), during (b), and after (c) the most rapid phase of Fe(II) oxidation. Dominant reactions in each panel are indicated by bold arrows, and minor/inhibited reactions are indicated by dashed arrows. (a) In the presence of acetate, respiratory complexes remain reduced by electrons from the quinone pool and enzymatic NO_3^-, NO_2^-, NO, and N_2O reduction proceeds. Periplasmic Fe(II) concentrations are low because of metal efflux pumping, and few reactions between Fe(II) and reduced respiratory complexes occur. Some extracellular reactions between Fe(II) and NO_2^-/NO may proceed, depending on how much NO_2^- and NO accumulates outside the cell. (b) Once acetate has dropped to a low level, Fe(II) will accumulate in the periplasm and undergo oxidation coupled to nitrogen oxide reduction at respiratory complexes and other periplasmic components in the most rapid phase of Fe(II) oxidation. (c) As Fe(II) is oxidized at respiratory complexes, mineral precipitates will begin to form in association with these proteins, leading to their inhibition and the accumulation of NO_2^-, NO, and N_2O. Extracellular NO_2^- and NO can oxidize solid-phase Fe(II), but NO can also solubilize Fe(II), which could diffuse back into cells to be oxidized. Ultimately, periplasmic Fe(III) precipitation damages respiratory complexes and other periplasmic sites, leading to a lower rate of NDFO, driven primarily by abiotic reactions.

reduction may be directly catalyzed by Nar, Nir, and Nor (54) or other periplasmic components or through abiotic reactions that can be accelerated by ligands (53). If the rate of Fe(II) oxidation and Fe(III) mineral precipitation is faster than the rate of efflux, precipitation of Fe(II) minerals on periplasmic proteins will lead to their inhibition and NO_2^-, NO, and N_2O accumulation (see Fig. S2d to f in the supplemental material) (13, 14, 62). In our study, NO_2^-, NO, and N_2O did not accumulate to their maximum steady-state level and acetate was not completely consumed in NDFO cultures or cell suspensions until after the more rapid phase of Fe(II) oxidation (Fig. 5; see Fig. S2b and d to f and S5). This result suggests that a catalyst of Fe(II) oxidation is depleted alongside a catalyst of NO_2^-, NO, and N_2O reduction. As no inducible Fe(II) oxidoreductase appears to be present in *A. ebreus* (see Data Set S1), the simplest explanation of our results is that an important site of Fe(II) oxidation is the same as the site of NO_2^-, NO, and N_2O reduction (i.e., the redox-active proteins Nar, Nir, Nor, and Nos and other redox-active components of the periplasm). A model of NDFO consistent with our results and results in the literature is presented in Fig. 6.

We postulate that the periplasmic components of all nitrate-reducing bacteria are innately capable of catalyzing a baseline level of NDFO. The rate and extent of NDFO will depend on differences in the respiratory chain components, the Fe(II) form, the accumulation of respiratory intermediates (NO_2^-, NO), the concentration of the organic cosubstrate, and the capacity of the bacteria to overcome Fe(II) and nitrogen oxide toxicity through efflux pumps and detoxification mechanisms. While an inducible Fe(II) oxidoreductase may exist, our results suggest that it is not necessary for a nitrate-reducing organism to be proficient at catalyzing NDFO.

Some previous work suggests a growth benefit from NDFO. In batch cultures, the effect is subtle (12, 14), but continuous flow may enhance the growth benefit (14). We did not observe a significant growth benefit due to NDFO in our batch cultures of *A. ebreus* (see Fig. S8 in the supplemental material), but it may be that any energy gain from Fe(II) oxidation is largely masked by the energy cost of coping with Fe(II) toxicity. We have recently proposed several possible mechanisms whereby an organism could gain an energy benefit from NDFO (3). Future work should focus on demonstrating which, if any, of these dominate in different bacteria.

Preliminary proteomic data suggest that efflux pumps are induced during NDFO in both *A. suillum* and *P. ferrooxidans*, but genetic knockouts and other experiments are needed to precisely define their role. Also, more studies of the reactivity of NO and NO_2^- with various geochemically important Fe(II) forms are needed. An awareness of the accumulation of NO is important to a thorough understanding of the influence of Fe(II) on biological systems. For example, recent work has emphasized the importance of NO in regulating bacterial motility and biofilm formation by environmentally important species (25, 63), including the model Fe(III) reducer *Shewanella oneidensis* (64), but almost nothing is known about the biogeochemical controls on NO production in the environment. Finally, both NO and N_2O are well-known atmospheric pollutants and the emissions of NO and N_2O from diverse environments have been compared (65, 66). However, to our knowledge, no study has assessed the importance of organic electron donors in controlling NO and N_2O emissions in Fe(II)-rich systems. Our results suggest that NDFO may be a major contributor to the flux of nitrogen oxide intermediates in Fe(II)-rich systems limited for organic electron donors.

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H.K.C., I.C.C., and J.D.C. designed the research. H.K.C., A.T.I., I.C.C., and S.J.B. performed research and analyzed data. H.K.C., I.C.C., and J.D.C. wrote the paper.

We have no conflict of interest to declare.

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