Denitrification by *Anaeromyxobacter dehalogenans*, a Common Soil Bacterium Lacking the Nitrite Reductase Genes nirS and nirK

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ABSTRACT

The versatile soil bacterium *Anaeromyxobacter dehalogenans* lacks the hallmark denitrification genes nirS and nirK (encoding NO₂⁻ → NO reductases) and couples growth to NO₃⁻ reduction to NH₄⁺ (respiratory ammonification) and to N₂O reduction to N₂. *A. dehalogenans* also grows by reducing Fe(III) to Fe(II), which chemically reacts with NO₂⁻ to form N₂O (i.e., chemodenitrification). Following the addition of 100 μmol of NO₃⁻ or NO₂⁻ to Fe(III)-grown axenic cultures of *A. dehalogenans*, 54 (±7) μmol and 113 (±2) μmol N₂O-N, respectively, were produced and subsequently consumed. The conversion of NO₃⁻ to N₂ in the presence of Fe(II) through linked biotic-abiotic reactions represents an unrecognized ecophysiology of *A. dehalogenans*. The new findings demonstrate that the assessment of gene content alone is insufficient to predict microbial denitrification potential and N loss (i.e., the formation of gaseous N products). A survey of complete bacterial genomes in the NCBI Reference Sequence database coupled with available physiological information revealed that organisms lacking nirS or nirK but with Fe(III) reduction potential and genes for NO₃⁻ and N₂O reduction are not rare, indicating that NO₃⁻ reduction to N₂ through linked biotic-abiotic reactions is not limited to *A. dehalogenans*. Considering the ubiquity of iron in soils and sediments and the broad distribution of dissimilatory Fe(III) and NO₃⁻ reducers, denitrification independent of NO-forming NO₂⁻ reductases (through combined biotic-abiotic reactions) may have substantial contributions to N loss and N₂O flux.

IMPORTANCE

Current attempts to gauge N loss from soils rely on the quantitative measurement of nirK and nirS genes and/or transcripts. In the presence of iron, the common soil bacterium *Anaeromyxobacter dehalogenans* is capable of denitrification and the production of N₂ without the key denitrification genes nirK and nirS. Such chemodenitrifiers denitrify through combined biotic and abiotic reactions and have potentially large contributions to N loss to the atmosphere and fill a heretofore unrecognized ecological niche in soil ecosystems. The findings emphasize that the comprehensive understanding of N flux and the accurate assessment of denitrification potential can be achieved only when integrated studies of interlinked biogeochemical cycles are performed.

KEYWORDS

*Anaeromyxobacter*, chemodenitrification, coupled Fe- and N-redox processes, denitrification, nitrogen loss

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F
ixed nitrogen (N) availability limits primary production in most natural and managed soil ecosystems, and to meet the demands of a growing human population for food, feed, and biofuel crops, global fertilizer usage continues to increase. The Haber-Bosch process (the chemical conversion of N$_2$ to ammonia [NH$_3$]) introduces about 100 Tg of fixed N into the environment each year worldwide as fertilizer (1). As a consequence, the mass of fixed N has doubled within the last century and is at levels never seen over the history of life on Earth. A substantial amount of the fixed N is lost from the soil due to microbial nitrification (ammonium oxidation to nitrate; NH$_4^+$→NO$_3^-$) and subsequent microbial denitrification (nitrate reduction to nitrous oxide/nitrogen gas; NO$_3^-$→N$_2$/O$_2$/N$_2$). Both nitrification and denitrification generate N$_2$O (2), a potent greenhouse gas with ozone depletion potential (3). To assess the role of microorganisms in N loss from soils to the atmosphere (i.e., emissions of the gaseous products N$_2$/O and N$_2$), the measurement of the denitrification genes nirS and nirK (encoding nitrite→nitric oxide [NO$_2^-$→NO] reductases) and nosZ (encoding N$_2$/O→N$_2$ reductase) has been applied. Several studies demonstrated a positive correlation between the abundance of these denitrification genes (i.e., nirK, nirS, and nosZ) and NO$_3^-$/NO$_2^-$ conversion to gaseous products (for examples, see references 4–7); however, other studies failed to establish this relationship (8–11). Subsequent efforts showed that the commonly used nirK and nirS quantitative PCR (qPCR) primers yield poor coverage of nirK and nirS sequences recovered from metagenomes and underestimate the true nirK and nirS gene abundance in environmental samples (12, 13). Similar issues apply to nosZ, and recent efforts discovered novel clade II nosZ genes, which are not amplified by previously designed primers targeting the clade I nosZ sequences (14, 15). The analysis of metagenomic data sets revealed that the previously unrecognized clade II nosZ genes are more prevalent in many soil ecosystems, suggesting that their hosts are potentially relevant contributors to N$_2$O consumption (14–17). Additionally, a multitude of environmental parameters, such as soil moisture (i.e., water-filled pore space), soil type, total N, redox potential (E$_{h}$), and reactive iron content, may also influence N$_2$/O emissions (9, 18, 19). Chemodenitrification, a process in which NO$_3^-$ is converted to N$_2$/O in a chemical (abiotic) redox reaction with ferrous iron [Fe(II)] (20), has not been attributed a major role in soil N loss. A few studies have shown that NO$_3^-$-reducing microorganisms couple chemodenitrification (NO$_3^-$→N$_2$/O) and biotic reactions (NO$_3^-$→NO$_2^-$) to reduce NO$_3^-$/NO$_2^-$ and oxidize Fe(II) (21–23). Recent efforts have demonstrated rapid chemodenitrification under a range of NO$_3^-$ and Fe(II) concentrations (24–26) and provided evidence for in situ chemodenitrification in soils and sediments (26, 27). These observations indicate that monitoring the nirS and nirK genes is insufficient to gauge denitrification potential, and abiotic factors should be considered for predicting N loss.

The soil bacterium Anaeromyxobacter dehalogenans strain 2CP-C reduces ferric iron [Fe(III)] to Fe(II) (28) and reduces NO$_3^-$ to NH$_4^+$ with NO$_2^-$ as the intermediate via the respiratory ammonification pathway (15, 29). A. dehalogenans possesses the narG and napA genes (encoding NO$_3^-$ reductases) and the nrfA gene (encoding NO$_3^-$→NH$_4^+$ reductase) and the nosZ gene (encoding clade II N$_2$/O→N$_2$ reductase), but the organism is not classified as a denitrifier due to the observed NO$_3^-$ to NH$_4^+$ reduction activity and the absence of nirK and nirS. Recent studies demonstrated that nosZ genes belonging to members of the Anaeromyxobacter genus compose a large proportion of the total nosZ genes in Illinois soil metagenomes (16) and that Anaeromyxobacter transcripts compose a sizeable proportion of nar, nor, nos, and nrf transcripts in rice paddy soils (30). Since little is known how organisms with genes encoding NO$_3^-$ and N$_2$/O reductases but lacking nirK and nirS might bridge the Fe and N cycles, the effect of Fe(II) on NO$_3^-$ and NO$_2^-$ reduction was explored in axenic A. dehalogenans cultures. These experiments demonstrated that A. dehalogenans facilitates chemodenitrification through Fe(III) and NO$_3^-$ reduction and ultimately denitrifies to N$_2$ through coupled biotic-abiotic reactions.
RESULTS

*A. dehalogenans* reduces NO$_3^-$ to N$_2$ and NH$_4^+$ in the presence of Fe(II). In the absence of Fe(III) as the electron acceptor, stoichiometric conversion of NO$_3^-$ to NH$_4^+$ was observed in pure cultures of *A. dehalogenans*, consistent with the organism’s classification as a respiratory ammonifier (29). In contrast, the addition of 100 μmol of NO$_3^-$ to *A. dehalogenans* strain 2CP-C cultures that had reduced 480 (±20) μmol of Fe(III) [as Fe(III) citrate] resulted in the rapid formation of 54 (±7) μmol N$_2$O-N and 63 (±5) μmol NH$_4^+$ (Fig. 1A). The N$_2$O was subsequently consumed (Fig. 1A) but was stable in replicate cultures containing acetylene, which inhibits N$_2$O reduction (Fig. 1B). qPCR enumeration of 16S rRNA genes revealed cell increases indicative of growth during the Fe(III) and NO$_3^-$/NO$_2^-$ reduction phases (Table 1; see Fig. S1 in the supplemental material). Although *A. dehalogenans* couples N$_2$O reduction with energy conservation and growth (15, 17), qPCR could not reveal additional cell increases (expected yield of 1.3 × 10$^7$ cells/ml from 54 μmol N$_2$O-N reduction after day 12) due to prior growth with Fe(III) and NO$_3^-$ as electron acceptors (resulting in 6.0 × 10$^7$ cells/ml) and interference of Fe(III) oxide precipitate in DNA extraction. A bright orange precipitate, presumably Fe(III) oxides, formed in the DNA extraction tubes, suggesting interference and reduced DNA extraction efficiency following Fe(III) reduction activity (31). In the cultures lacking Fe(II) [i.e., containing only the 750 nmol of Fe(II) introduced with the trace metal solution], *A. dehalogenans* reduced 200 (±10) μmol NO$_3^-$ to 190 (±12) μmol NH$_4^+$ (Fig. S2), which is consistent with the gene content of *A. dehalogenans* and the organism’s characterization as a respiratory ammonifier (15, 29). The amount of N$_2$O-N did not exceed 2 μmol in any of the incubation vessels not amended

![Figure 1](http://aem.asm.org/)

FIG 1 Abiotic production of N$_2$O after NO$_3^-$ addition to *A. dehalogenans* cultures grown with Fe(III). (A) *A. dehalogenans* grown in defined mineral salts medium with 1,000 μmol of acetate and 700 μmol of Fe(III) citrate. NO$_3^-$ (100 μmol) was added on day 10 (indicated by an arrow in each panel). (B) Same growth conditions as those described for panel A except that acetylene (10% of headspace) was included to prevent NosZ activity. The error bars represent the standard deviation of results of three replicate cultures. Symbols: black squares, Fe(II); open circles, N$_2$O-N; open triangles, NH$_4^+$. No error bars are shown if the standard deviation is smaller than the symbol.
with Fe(III) as the electron acceptor. Thus, the major product of NO_3^-/H_11002^+ reduction was NH_4^+ in the absence of Fe(II) and N_2 in the presence of Fe(II).

**A. dehalogenans reduces NO_2^- to N_2 in the presence of Fe(II).** Similar to NO_3^- amendment, the addition of 100 μmol of NO_2^- to A. dehalogenans strain 2CP-C cultures that had reduced 590 (±60) μmol of Fe(III) resulted in rapid N_2O formation (Fig. 2A). N_2O formation also occurred in bottles that were heat treated prior to NO_2^- addition, indicating that the reduction of NO_2^- to N_2O was an abiotic process (Fig. 2B).

![FIG 2](image-url) Abiotic production of N_2O after NO_2^- addition to A. dehalogenans cultures grown with Fe(III). (A) A. dehalogenans grown in defined mineral salts medium with 100 μmol of acetate and 900 μmol of Fe(III) citrate. On day 14, 100 μmol of NO_2^- was added (indicated by the left downward arrow in each panel). On day 18, an additional 200 μmol of acetate was added (indicated by the right downward arrow in each panel). (B) Cultures treated the same as described for panel A except that they were autoclaved prior to NO_2^- addition. Error bars represent the standard deviation of results of three replicate cultures. Symbols: black squares, Fe(II); open circles, N_2O-N. No error bars are shown if the standard deviation is smaller than the symbol.

**TABLE 1** qPCR data showing cell growth associated with Fe(III), NO_3^-, and N_2O reduction, corresponding to Fig. 1 and 2a

<table>
<thead>
<tr>
<th>Corresponding figure</th>
<th>A. dehalogenans (cells ml^-1 (SD)^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial^c</td>
</tr>
<tr>
<td>1A</td>
<td>4.8 × 10^3 (1.5 × 10^3)</td>
</tr>
<tr>
<td>1B</td>
<td>7.8 × 10^3 (3.2 × 10^3)</td>
</tr>
<tr>
<td>2A</td>
<td>2.2 × 10^4 (1.4 × 10^4)</td>
</tr>
<tr>
<td>2B</td>
<td>2.1 × 10^4 (1.4 × 10^4)</td>
</tr>
</tbody>
</table>

^aSee Fig. S1 and S3 in the supplemental material for more detailed qPCR data.

^bStandard deviations of triplicate cultures are indicated in parentheses.

^cCells introduced with the inoculum.

^dDay 10 for Fig. 1A and B; day 13 for Fig. 2A and B.

^eDay 12 for Fig. 1A and B; day 18 for Fig. 2A and B.

^fDay 21 for Fig. 1A; day 41 for Fig. 1B.

^gNA, not applicable.
In live and killed control incubations, 113 (±2) and 109 (±9) µmol N₂O-N were produced, respectively, indicating stoichiometric conversion of NO₂⁻ to N₂O.

Live cultures had depleted the initial amount of 100 µmol acetate during the initial Fe(III) reduction phase, and N₂O was not consumed. Following the addition of 200 µmol of acetate on day 18, Fe(III) reduction resumed and N₂O consumption started (Fig. 2A). During the initial Fe(III) reduction phase, 4.3 × 10⁶ cells/ml were produced, and an increase by approximately 1.2 × 10⁷ cells/ml was measured during the consumption of N₂O as the electron acceptor (Fig. S3). The cell yield is slightly lower than the expected value of 2.7 × 10⁷ cells/ml (for 113 µmol N₂O-N) (Table 1), which is likely due to interferences of iron precipitates with DNA extraction and subsequent qPCR enumeration (see above). In controls that were heat killed following the initial Fe(III) reduction phase, the biotically produced Fe(II) reacted with NO₂⁻, leading to the formation of stoichiometric amounts of N₂O, but N₂O was stable even after acetate addition, indicating that live cells were required for N₂O reduction to occur (Fig. 2B). Live and heat-killed cultures produced N₂O at similar rates of 1.9 (±0.03) and 1.9 (±0.13) µmol h⁻¹, respectively (Fig. 2A and B), whereas lower N₂O formation rates of 1.3 (±0.04) µmol h⁻¹ were measured in abiotic (i.e., no cells) control incubations with 800 µmol of ferrous chloride and 100 µmol of NO₂⁻ (Fig. S4). These findings suggest that equimolar concentrations of biotically produced Fe(II) were more reactive than Fe(II) added as ferrous chloride.

**N₂O formation in cultures with insoluble Fe(III) and effect of sulfide on N₂O reduction.** NO₂⁻ reduction was also monitored in *A. dehalogenans* cultures that had reduced 250 (±7) µmol poorly crystalline Fe(III) oxyhydroxide (Fig. 3) or 960 (±50) µmol soluble Fe(III) (as ferric citrate; data not shown) in medium containing 0.2 mM sulfide as a reducing agent. With insoluble and soluble forms of Fe(III), 35 (±4) and 47 (±8) µmol N₂O-N were produced, respectively, during the reduction of 100 µmol NO₂⁻, but N₂O was not subsequently consumed by *A. dehalogenans* (Fig. 3). Cultures of *A. dehalogenans* consistently used N₂O as a respiratory electron acceptor in defined, anoxic medium without sulfide (Fig. S5), and further tests were performed to determine if the presence of sulfide could explain why N₂O was not consumed. In medium amended with 0.2 mM sulfide, *A. dehalogenans* cultures did not reduce N₂O (Fig. S5), despite the ability of *A. dehalogenans* to grow with other electron acceptors [e.g., NO₃⁻ and Fe(III)] in the presence of 0.2 mM sulfide.

**Denitrification potential of bacteria lacking NO-forming NO₂⁻ reductases.** The NCBI Reference Sequence (RefSeq) database was searched for organisms that have at least one nitrate reductase gene (*napA* and/or *narG*) and *nosZ* genes and lack *nirK* and *nirS* genes. If also capable of Fe(III) reduction, these organisms could potentially occupy...
a previously unrecognized ecological niche as complete denitrifiers (NO$_3^-$→N$_2$) without the need for NO- forming NO$_2^-$ reductases. Among 4,739 translated RefSeq genomes examined, 336 genomes had at least one nitrate reductase gene (napA or narG) and one N$_2$O reductase gene (nosZ) but lacking NO$_2^-$ reductase (nirK or nirS) genes. All intergeneric nodes with bootstrap values of <50% are marked with red circles. Four archaeal sequences were included as outgroups, and the branch coloring reflects class taxonomy. For all bacteria, the estimated number of c-type cytochromes (with the number of c-type cytochromes with >4 predicted heme-binding domains in parentheses), demonstrated ability to reduce Fe(III) (indicated by a plus sign), the type of nosZ gene (i.e., clade I or clade II), and the source of the isolate are indicated. The absence of a plus sign for Fe(III) reduction indicates that this activity is unknown (most frequently, untested), and the absence of a symbol in the source column indicates that the source is unknown. The scale bar corresponds to the number of nucleotide substitutions per site. References for individual isolates are provided in Table S1 in the supplemental material. The Venn diagram shows the proportions of the analyzed RefSeq genomes containing nirK or nirS, napA or narG, and nosZ. Out of the 4,739 genomes analyzed, 2,567 had either a napA or a narG gene, 634 had a nirK or a nirS gene, and 394 had a nosZ gene.* isolated from an alkaline lake.
to reduce Fe(III), the genomes were further searched for c-type cytochromes with multiple heme-binding domains, a shared trait among many Fe(III)-reducing bacteria (33). All 51 genomes that fit the above-mentioned criteria contain 15 or more putative c-type cytochromes, and in 27 genomes, at least six of the c-type cytochromes have four or more predicted heme-binding domains (Fig. 4). Of course, these criteria are insufficient to prove that these organisms are indeed Fe(III) reducers, and physiological studies are required. Still, these observations suggest that a much broader organismal group not possessing nirS or nirK has the potential to perform denitrification by coupling biotic and abiotic reactions. The term chemodenitrifier is proposed to describe organisms that combine the chemical chemodenitrification reactions [i.e., Fe(II)-mediated NO$_2^-$/$\text{H}_2\text{O}_2$ conversion] and an enzymatic reaction(s) to reduce NO$_3^-$ to N$_2$O or N$_2$ (i.e., denitrification that is independent of NO-forming NO$_3^-$ reductases). The organisms uncovered by the above analysis represent potential chemodenitrifiers, as they lack the keystone denitrification genes nirK and nirS yet have the potential to reduce NO$_3^-$ to N$_2$. Of note, not included in this analysis are potential chemodenitrifiers that lack nosZ and potential chemodenitrifiers that possess nirK or nirS but reduce NO$_2^-$ to N$_2$O via Fe(II)-mediated rather than enzymatic reactions.

**DISCUSSION**

This study reveals a heretofore unrecognized ecological niche for *A. dehalogenans* and organisms with shared physiological features that directly link the N and Fe cycles. Previous growth studies characterized *A. dehalogenans* as a NO$_3^-$-reducing respiratory ammonifier that is also able to reduce N$_2$O to N$_2$ by utilizing a clade II NosZ (15, 29). Genome analysis corroborated these observations, and no genes responsible for NO$_2^-$ reduction to NO (i.e., nirS or nirK) occur on the genome (34). Prior pure culture studies were performed with single electron acceptors, and potential synergistic effects were not recognized. The results presented here demonstrate that Fe(III) reduction profoundly affects NO$_3^-$ and NO$_2^-$ metabolism in *A. dehalogenans* (Fig. 5). Complete NO$_3^-$ reduction to N$_2$ was the result of a combination of biotic and abiotic reactions in *A. dehalogenans* cultures, indicating that cultivation on single electron acceptors and genome analysis failed to show that *A. dehalogenans* effectively acts as a denitrifier when Fe(III) and N oxides are present.

Combined biotic-abiotic reactions have been demonstrated in other NO$_3^-$-reducing cultures, including cultures of *Escherichia coli*, *Shewanella putrefaciens* strain 200, *Wolinella succinogenes*, and *Klebsiella mobilis*. NO$_3^-$-reducing cultures of *E. coli* were shown to abiotically produce N$_2$O through chemodenitrification when Fe(II) was present in the medium (35). Cultures of *S. putrefaciens* were also shown to produce N$_2$O during NO$_3^-$ and Fe(III) reduction (21). N$_2$O was the end product in experiments with *E. coli* and *S.
TABLE 2  Free energy changes (ΔG′) associated with different N and Fe cycling processes at pH 7.0 and 30°C

<table>
<thead>
<tr>
<th>Equation</th>
<th>Reaction</th>
<th>Free energy changes (kJ/reaction)</th>
<th>Free energy boundaries for total reactions (kJ/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NO₃⁻ + 0.25 CH₃COO⁻ → NO₂⁻ + 0.25 H⁺ + 0.5 HCO₃⁻</td>
<td>-152.5 (AC)</td>
<td>-137.1 (BD)</td>
</tr>
<tr>
<td>2</td>
<td>2 Fe³⁺ (aq) + H₂O + 0.25 CH₃COO⁻ → 2.25 H⁺ + 2 Fe²⁺ (aq) + 0.5 HCO₃⁻</td>
<td>-247.8 (GC)</td>
<td>-206.2 (HD)</td>
</tr>
<tr>
<td>3</td>
<td>NO₂⁻ + 2 Fe²⁺ (aq) + 4.5 H₂O → 0.5 N₂(g) + 3 H⁺ + 2 Fe(OH)₃(amorph)</td>
<td>-169.6 (BHE)</td>
<td>-108.4 (AGF)</td>
</tr>
<tr>
<td>4</td>
<td>2 Fe(OH)₃(amorph) + 3.75 H⁺ + 0.25 CH₃COO⁻ → 5 H₂O + 2 Fe²⁺ (aq) + 0.5 HCO₃⁻</td>
<td>-52.3 (GC)</td>
<td>-52.3 (HD)</td>
</tr>
<tr>
<td>5</td>
<td>0.5 N₂O₃(g) + 0.125 CH₃COO⁻ → 0.5 N₂(g) + 0.125 H⁺ + 0.25 HCO₃⁻</td>
<td>-136.8 (ED)</td>
<td>-154.2 (FD)</td>
</tr>
<tr>
<td>6</td>
<td>NO₃⁻ + 0.75 CH₃COO⁻ + 1.25 H⁺ + H₂O → NH₄⁺ + 1.5 HCO₃⁻</td>
<td>-386.1 (BC)</td>
<td>-363.2 (AD)</td>
</tr>
<tr>
<td>7</td>
<td>NO₃⁻ + 0.875 CH₃COO⁻ + 2 Fe²⁺ (aq) + 0.5 H₂O → 0.5 N₂(g) + 2 Fe³⁺ + 1.75 HCO₃⁻ + 1.875 H⁺</td>
<td>-606.8</td>
<td>-532.4</td>
</tr>
<tr>
<td>8</td>
<td>NO₃⁻ + 0.875 CH₃COO⁻ + 2 Fe(OH)₃(amorph) + 4.125 H⁺ → 0.5 N₂(g) + 2 Fe²⁺ + 1.75 HCO₃⁻ + 5.5 H₂O</td>
<td>-411.3</td>
<td>-378.5</td>
</tr>
<tr>
<td>9</td>
<td>NO₃⁻ + CH₃COO⁻ + 1.0 H⁺ + H₂O → NH₄⁺ + 2 HCO₃⁻</td>
<td>-538.6</td>
<td>-500.3</td>
</tr>
</tbody>
</table>

*aInitial and final ΔG′ values were calculated in Geochemists Workbench (Aqueous Solutions, LLC). Initial free energy change values were calculated using the concentrations of reactants/products present at the beginning of the reactions. Final free energy change values were calculated using the concentrations of reactants/products measured or estimated at the end of the reactions. Bold values indicate an abiotic reaction not directly coupled to microbial energy metabolism.

bThe total free energy changes associated with complete NO₃⁻ conversion to N₂ (equations 7 and 8) or NH₄⁺ (equation 9) were calculated by adding the ΔG′ values for the individual reactions (equations 1 to 6). The values reported as maximums represent the highest possible free energy change (i.e., most-negative ΔG′ values) associated with the respective reactions, while the values reported as minimums represent the lower threshold for free energy changes (i.e., the most-positive ΔG′ values). To calculate the maximum free energy values, the largest (i.e., most-negative) free energy change values from the individual reactions (equations 1 to 6) were added together. To calculate the minimum free energy values, the smallest (i.e., most-positive) free energy change values were added. Note that the ΔG′ value for Fe²⁺ was added twice in equation 8 to account for amorphous Fe³⁺ produced during chemodenitrification. Equation 7 was calculated by adding equations 1, 2, 4, and 5. Equation 8 was calculated by adding equations 1, 4 (twice), and 5. Equation 9 was calculated by adding equations 1 and 6.

putrefaciens strain 200, in contrast to the experiments with A. dehalogenans, in which NO₃⁻ was completely reduced to N₂. The formation and consumption of small amounts of N₂O (0.15% of the NO₃⁻-N) were observed in NO₃⁻-replete cultures of W. succinogenes, a respiratory ammonifier possessing a clade II nos operon (36). Like Anaeromyxobacter, W. succinogenes lacks nirK and nirS, and it was speculated N₂O was produced through reactions of NO₃⁻ with medium components and NO detoxification (36). In the presence of Fe(III) and NO₃⁻, A. dehalogenans produced substantial amounts of N₂O (i.e., greater than 50%) from available NO₃⁻ or NO₂⁻ and has the enzymatic capacity of subsequently reducing the N₂O to N₂. In contrast to studies with E. coli, S. putrefaciens, and W. succinogenes, the work with Anaeromyxobacter demonstrates that a bacterium lacking NO-forming NO₂⁻ reductases can effectively reduce NO₃⁻/NO₂⁻ to N₂ via coupled biotic-abiotic reactions. It should be noted that studies with K. mobilis demonstrated abiotic NH₄⁺ production via NO₂⁻ reaction with green rust-associated Fe(II) (23); however, evidence for abiotic NH₄⁺ production was not observed in any of the incubation studies with A. dehalogenans.

Due to a versatile metabolism, A. dehalogenans is an ecologically competitive bacterium in various ecosystems (15, 29, 37). Previous studies uncovered the novel clade II nosZ genes and demonstrated efficient growth of A. dehalogenans coupled to N₂O reduction (15, 17). The clade II nos operons provide greater growth yields (i.e., more efficient energy capture), and bacteria expressing clade II NosZ exhibit greater affinity to N₂O than bacteria with clade I NosZ, suggesting that organisms with clade II NosZ outcompete organisms with clade I NosZ at low (i.e., environmentally relevant) N₂O concentrations (17). In the presence of Fe(III), chemodenitrification prevents A. dehalogenans from effectively coupling growth with NO₂⁻ reduction to NH₄⁺; however, the organism has the potential to conserve energy from N₂O to N₂ reduction (15, 17). Respiratory ammonification and complete NO₃⁻ reduction to N₂ (via combined biotic reactions and chemodenitrification) are both associated with similar negative Gibbs free energy changes (ΔG′) (Table 2), suggesting that both of these pathways are
equally favorable from an energetic perspective. *A. dehalogenans* captures more energy on a per electron basis from N₂O to N₂ reduction than from NO₂⁻ to NH₄⁺ reduction (i.e., the reduction of 0.5 mol of N₂O yields 2.4 × 10¹³ cells compared to a yield of 2.0 × 10¹³ cells from the reduction of 0.16 mol of NO₂⁻) (Table 3, equations 3 and 1, respectively). Additionally, the reaction between NO₂⁻ and Fe(II) yields Fe(III), which serves as an electron acceptor for *A. dehalogenans*. By coupling respiratory [NO₃⁻→NO₂⁻, N₂O→N₂, and Fe(III)→Fe(II)] and abiotic [NO₂⁻→N₂O, Fe(II)→Fe(III)] reactions (Fig. 5), *A. dehalogenans* has the potential to produce more biomass than it does via respiratory ammonification (NO₃⁻→NH₄⁺), demonstrating an ecological strategy to optimize energy capture and competitiveness. Collectively, the experiments presented here and past N₂O growth experiments (15, 17) demonstrate that the nondenitrifier *A. dehalogenans* can reduce NO₃⁻ to N₂ in the presence of Fe(III)/Fe(II), and the organism potentially benefits from this coupled biotic-abiotic process by conserving more energy than it does via the respiratory ammonification pathway.

An initially perplexing observation was the inability of *A. dehalogenans* to grow with N₂O produced by abiotic reactions (see Fig. S5 in the supplemental material) when the medium received sulfide as a reducing agent. No such growth inhibition was observed with NO₂⁻ and ferric iron as electron acceptors, suggesting that the observed growth inhibition was due to the sequestration of copper (38). The requirement of copper for N₂O reductases suggests that chemodenitrifiers such as *A. dehalogenans* contribute to N₂O production in sulfidic (that is, copper-limited) environments (Fig. 5).

The coupled biotic-abiotic reactions in NO₃⁻- and Fe(III)-reducing *A. dehalogenans* cultures reveal an important ecological role for organisms that possess nos operons. Fe(II) and NO₂⁻ cooccur in many natural environments, allowing interactions between N and Fe cycling (for an example, see reference 65) and suggesting that microbially mediated chemodenitrification occurs broadly. Soils contain 100 to 100,000 ppm iron, and in addition to Fe(III) serving as an electron acceptor in anoxic soils, Fe(III) and Fe(II) cycling commonly occurs near oxic-anoxic interfaces, thus replenishing the Fe(III) pool for dissimilatory Fe(III) reduction (40–43). Dissimilatory Fe(III)-reducing bacteria such as *A. dehalogenans* and *Geobacter* spp. are ubiquitous in soils and subsurface environments (44, 45). During NO₂⁻ reduction and ammonia oxidation, NO₂⁻ is released temporarily (Fig. S2) (46, 47), and NO₂⁻ can accumulate for weeks to months in both alkaline and neutral soils (48, 49). Additionally, the enzyme kinetics of NO₃⁻ and NO₂⁻ reductases vary by organism, and for organisms that reduce NO₃⁻ at higher rates than NO₂⁻, intermediate NO₂⁻ forms prior to further NO₂⁻ reduction (46). Fe(III) reduction occurs outside the cell or in the periplasm (50), and NO₂⁻ reduction occurs in the periplasm, suggesting that reactions with NO₂⁻ can occur even under conditions where NO₂⁻ does not accumulate extracellularly. When NO₂⁻ and Fe(II) cooccur outside the cytoplasm, enzymatic NO₂⁻ reduction must compete with abiotic chemistry (i.e., chemodenitrification). The competition between abiotic and biotic reduction of NO₂⁻ is evidenced by the mixed end products (i.e., N₂O and NH₄⁺) produced during NO₃⁻ reduction by *A. dehalogenans* (Fig. 1). The occurrence of Fe(II) and NO₂⁻ outside the cell suggests that microbially mediated chemodenitrification is not limited to organisms that can reduce both Fe(III) and NO₃⁻. A microbial community with Fe(III)- and

**TABLE 3** Theoretical growth yields per mole of electrons

<table>
<thead>
<tr>
<th>Equation</th>
<th>Process</th>
<th>Reduction half reaction</th>
<th>Growth yield (no. of cells/mol e⁻)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NO₂⁻ reduction to NH₄⁺</td>
<td>0.16 NO₂⁻ + 1 e⁻ + 4.33 H⁺ → 0.16 NH₄⁺ + 0.33 H₂O</td>
<td>2 × 10¹³</td>
<td>R. A. Sanford, unpublished data</td>
</tr>
<tr>
<td>2</td>
<td>Fe(III) reduction</td>
<td>Fe³⁺ + 1 e⁻ → Fe²⁺</td>
<td>6.0 × 10¹²</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>N₂O reduction</td>
<td>0.5 N₂O + 1 e⁻ + 1 H⁺ → 1/2 N₂ + H₂O</td>
<td>2.4 × 10¹³</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Chemodenitrification</td>
<td></td>
<td>1.5 × 10¹³</td>
<td>This study³</td>
</tr>
</tbody>
</table>

³Based on *Anaeromyxobacter dehalogenans* strain R cell enumeration with flow cytometry.

⁴Based on published numbers [1.4 mg biomass/mmol Fe(III) and 11.2 mg biomass/mmol N₂O] and a weight of 2.4 × 10⁻¹⁵ g per bacterial cell.

⁵Theoretical calculation based on the reduction of 0.5 mol Fe(III) and 0.25 mol N₂O (produced by chemical reaction between NO₂⁻ and Fe(III)).
NO$_3^-$-reducing microorganisms could facilitate chemodenitrification, especially under conditions where NO$_2^-$ is not immediately consumed following NO$_3^-$ reduction. As NO$_3^-$-reducing microorganisms generate NO$_2^-$, NO$_2^-$ can react with Fe(II) produced by Fe(III) reducers. Chemodenitrification results in the loss of NO$_2^-$ as an electron acceptor for microbial metabolism; however, organisms with N$_2$O reductases, in particular those that express high-affinity clade II NosZ (17), would be able to utilize the resulting N$_2$O as an electron acceptor. The possession of the nosZ gene would therefore confer an ecological advantage in anoxic environments with dissimilatory Fe(III)-reducing activity.

Chemodenitrifiers such as A. dehalogenans fill a previously unrecognized ecological niche. Chemodenitrification merely describes the abiotic decomposition of NO$_2^-$ coupled to Fe(II) oxidation, whereas the term chemodenitrifier describes a microorganism that reduces NO$_3^-$ to N$_2$O or N$_2$ via combined enzymatic and abiotic steps. These organisms may be (i) true denitifiers that possess nirS or nirK but reduce NO$_3^-$ to N$_2$O abiotically in the presence of Fe(II), or (ii) nondenitrifiers (i.e., organisms that lack nirS or nirK genes) that can denitrify only in the presence of Fe(II). Past studies have examined the cooccurrence of nirK, nirS, and nosZ in sequenced genomes, demonstrating that denitrification is a modular pathway and many genomes contain nosZ but lack nirK and nirS (15, 32). The observation of chemodenitrification in A. dehalogenans cultures reveals a possible ecophysiological explanation for nondenitrifiers that possess nosZ. The cooccurrence study of nirK, nirS, nosZ, and napA and narG (the last two of which were not included in the above-mentioned studies) revealed that nondenitrifiers (i.e., genomes lacking nirS and nirK) possess nosZ, napA and/or narG, and multiple genes encoding multiheme proteins [i.e., may have the Fe(III)-reducing phenotype] (Fig. 4; Table S1). Although a general set of biomarkers for Fe(III) reduction has not yet emerged, a suite of c-type cytochromes (typically with four or more heme binding sites) are indicated in Fe(III) reduction activity by at least some Fe(III)-reducing bacteria (33, 50). The identified potential chemodenitrifiers span multiple bacterial classes occurring in diverse habitats, suggesting that chemodenitrifiers are dispersed across the phylogenetic tree and represent previously unrecognized contributors to NO$_3^-$ reduction to N$_2$. The chemodenitrifier potential is a prediction based on genome sequence analysis, and experimental efforts are needed to confirm this physiology [that is, NO$_3^-$/Fe(III) reduction activity] for the organisms included in Fig. 4. Additionally, not all Fe(III)-reducing bacteria require c-type cytochromes (39), suggesting that the prediction of potential iron-reducing bacteria by searching for c-type cytochromes is a conservative approach and likely an underestimation of potential chemodenitrifiers. Physiological studies and measurements of the NO$_3^-$ reduction products of potential chemodenitrifiers will reveal if these organisms indeed utilize the chemodenitrifier ecological strategy demonstrated for A. dehalogenans.

Collectively, our findings show the reduction of NO$_3^-$ to N$_2$ mediated by a single organism via combined abiotic and biotic mechanisms in the absence of nirS and nirK (Fig. 5). The findings emphasize that gene content is not sufficient to predict the final products of NO$_3^-$ reduction, putting into question attempts to link denitrification activity with the abundance and expression of nirS and nirK. Chemodenitrifiers represent understudied contributors to the formation of gaseous products from N-oxyanions, and the study of chemodenitrifiers may impact our current understanding of N loss to the atmosphere. Nondenitrifiers lacking nirS or nirK but possessing napA, narG, and/or nosZ contribute to N$_2$O flux via coupled biotic-abiotic processes, and a holistic understanding of N flux can be achieved only when integrated studies of interlinked biogeochemical cycles are performed.

**MATERIALS AND METHODS**

**Chemicals.** HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) (≥99%), L-cysteine hydrochloride monohydrate (98.5 to 101.0%), anhydrous sodium acetate (≥99.2%), sodium hydroxide pellets (≥99.0%), sodium nitrite (≥99.6%), ammonium chloride, ferric chloride hexahydrate (≥99.7%), and ferrous ammonium sulfate hexahydrate (101.2%) were purchased from Fisher Scientific (Fair Lawn, NJ). N$_2$O (99%), Fe(III) citrate, and ferrous chloride tetrahydrate (≥99.0%) were purchased from Sigma-Aldrich.
Microbial Technologies, Ochelata, OK) as described previously (51) with the following modifications: defined mineral salts medium in 160-ml glass serum bottles sealed with butyl rubber stoppers (GeoCultures N2O-N, and any N2O-N that was removed during liquid and headspace sampling. N2O production rates decreased from 5.6 mM to 3 mM to allow more accurate quantification. The amount of NH4 oxyhydroxide, which were inoculated from fumarate-grown cultures (29). Sulfide (0.2 mM) was initially the stoppers facing upward. Cultures grown with the same electron donors and acceptors as the vessels, 10% of the 60-ml N2 headspace was replaced with acetylene to inhibit N2O conversion to N2 (52). In abiotic (i.e., no inoculum) control vessels, ferrous chloride and NO3 were added to final concentrations of 8 mM and 1 mM, respectively. All vessels were incubated without shaking at 30°C in the dark with the stoppers facing upward. Cultures grown with the same electron donors and acceptors as the experimental cultures were used as seed cultures, with the exception of cultures amended with Fe(III) oxyhydroxide, which were inoculated from fumarate-grown cultures (29). Sulfide (0.2 mM) was initially included as a reducing agent in cultures that received Fe(III) oxyhydroxide as the electron acceptor. All experiments used triplicate culture vessels, unless indicated otherwise, and were repeated independently at least twice.

The Fe(III) citrate stock solution was prepared by adding 24.5 g of ferric citrate to approximately 90 ml of a 1.7 M NaOH solution. The solution was boiled until it turned dark, translucent brown. After cooling, the resulting acidic solution was neutralized (pH 7.0) by slowly adding approximately 10 ml of 10 M NaOH. The final stock solution was autoclaved and contained approximately 0.6 M Fe(III) and 0.1 M Fe(II), as determined by the ferrozine assay (53). Poorly crystalline Fe(III) oxide was prepared from ferric chloride hexahydrate as described previously (45). Fe(III) oxide stocks were sterilized by daily heating in a 90°C water bath and overnight cooling at room temperature over a 5-day period. A ferrous chloride stock solution was prepared inside an anoxic chamber (Coy Laboratory Products, Grass Lake, MI) by adding 9.9 g of ferrous chloride tetrahydrate to 100 ml of ultrapure water degassed with N2, and the green solution was stored in N2-gassed, sealed glass serum bottles after filter sterilization.

Analytical methods. N2O in the headspace of the incubation vessels was quantified with a gas chromatograph as described previously (15, 54). To account for cooling during sample extraction (due to removal from the 30°C incubator), the temperature of an uninoculated medium bottle was measured with a glass thermometer and used to select Ostwald coefficients of 0.6788, 0.5937, and 0.5241 for temperatures of 20°C, 25°C, and 30°C, respectively (55). Total N2O-N (i.e., the total N2O mass multiplied by a factor of two) in culture vessels is reported as the sum of the aqueous phase N2O-N, the headspace N2O-N, and any N2O-N that was removed during liquid and headspace sampling. N2O production rates were calculated by graphing N2O-N over time to determine the linear range of N2O production and are reported in micromoles per hour. Acetate was quantified by high-performance liquid chromatography (1200 series; Agilent Technologies, Santa Clara, CA) using an organic acid analysis column (Aminex HPX-87H ion exclusion column; Bio-Rad, Hercules, CA) heated to 30°C. The eluent was 4 mM sulfuric acid prepared in Milli-Q water, and the flow rate was 0.6 ml min⁻¹.

NH4⁺ was quantified with an ion chromatograph ( Dionex ICS-1100) and a Dionex IonPac cation exchange CS16 column heated to 30°C. The eluent was 20 mM sulfuric acid, and the flow rate was 1 ml min⁻¹. NO3⁻ and NO2⁻ were quantified by ion chromatography using a reagent-free eluent regeneration system (ICS-2100; Dionex, Sunnyvale, CA) and a Dionex IonPac AS18 4+ by 250-mm analytical column heated to 30°C. The eluent was 10 mM KOH, and the flow rate was 1 ml min⁻¹. Acid-extractable Fe(III)/Fe(II) were measured using a modification of the ferrozine assay (53). Briefly, ferrozine buffer was prepared with 1 g/liter Acros Organics FerroZine iron reagent dissolved in 50 mM HEPES buffer (56). Cultures were shaken vigorously by hand, suspensions were withdrawn by syringe, and 100 or 500 μl was incubated overnight in 10-ml glass vials containing 5 ml of 0.04 M sulfamic acid, which was used to prevent a chemical reaction of NO2⁻ with Fe(II) (22). After the vials were shaken, 20-μl aliquots were withdrawn and mixed with 1 ml of ferrozine buffer. Absorbance was measured using a Thermo Scientific Spectronic 20D spectrophotometer at a wavelength of 562 nm. Samples were measured quickly after mixing with the ferrozine buffer to avoid interference with Fe(III) (56). Standards were prepared with ferrous ammonium sulfate hexahydrate spanning a concentration range of 1.0 mM to 20 mM Fe(II). For experiments in which NO2⁻ was added to Fe(III) citrate-grown cultures, 0.5 M HCl was used in lieu of sulfamic acid, and Fe(III) concentrations are not reported when NO2⁻ was present in the culture due to potential reactions between Fe(III) and NO2⁻ after sampling between Fe(II) and NO3⁻.

Cell enumeration. Genomic DNA was extracted using the protocol provided in the MoBio PowerSoil DNA isolation kit (Carlsbad, CA). Cells were enumerated with an established TaqMan qPCR assay (37) using the Applied Biosystems ViiA 7 real-time PCR system (Foster City, CA). Results were analyzed with the ViiA 7 software (version 1.2.3).
Genomic and phylogenetic analyses. Prokaryotic genomes (4,739) were searched for genes encoding N cycle enzymes (i.e., NarG, NapA, NirK, NirS, and NosZ) by performing a Hidden Markov Model (HMM) search with HMMER 3.1b2 [https://www.hmmer.org/]. HMMs for NarG, NapA, NirK, NirS, and NosZ were downloaded from FunGene [57]. The sequences of annotated proteins of available complete bacterial genomes (i.e., labeled as “Latest” and “Complete Genome”) from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database were downloaded in April 2016 and used as the target files. HMMER was run locally with a target E value cutoff of $1 \times 10^{-10}$ using a BASH script. Results were parsed and analyzed using Python [https://www.python.org/]. False positives were removed by searching for the absence of conserved domains visually in Geneious software suite version 8.1.9 [58] and with the Reverse Position-Specific Basic Local Alignment Tool (RPS-BLAST) [59]. The NCBI Conserved Domains Database (CDD) was downloaded in September 2016 from the NCBI FTP website (ftp://ftp.ncbi.nlm.nih.gov/pub/mmb/cdd/little_endian/Cdd_LE.tar.gz) for a local RPS-BLAST. Proteins that did not match conserved domains with an E value of $<1 \times 10^{-30}$ were excluded from further analysis.

Available bacterial 16S rRNA gene sequences of organisms containing narG, napA, and nosZ genes, but lacking nirK and nirS genes, and four outgroup archaeal 16S rRNA genes were downloaded from NCBI and used to construct a phylogenetic tree. Full-length 16S rRNA gene sequences were aligned with the SILVA Incremental Aligner version 1.2.11 [60] using default settings with unaligned bases removed from the alignment ends. Aligned positions containing only gaps were manually removed in Geneious. JModelTest 2 [61] was used to predict the optimal evolutionary model for tree reconstruction. A maximum likelihood tree was constructed with 100 bootstrap replicates with PhyML 3.0 [62] using a generalized time reversible (GTR) model [63] with gamma distributed rate heterogeneity and a proportion of invariable sites.

The organisms represented in the phylogenetic tree were searched in the literature for the ability to reduce Fe(III). The numbers of c-type cytochrome genes per genome were approximated by using a Python script modeled after a published script (64), which identified the heme binding motifs CxxCH, CxxxCH, and CxxxxCH in the annotated proteins of RefSeq genomes. Candidate c-type cytochromes were further screened by running PSI-BLAST against the nr NCBI database (downloaded in December 2016) and eliminating any sequences that did not match annotated c-type cytochromes with an E value of $<1 \times 10^{-11}$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01985-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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