# Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils

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**Agricultural and industrial practices more than doubled the intrinsic rate of terrestrial N fixation over the past century with drastic consequences, including increased atmospheric nitrous oxide ($N_2O$) concentrations. $N_2O$ is a potent greenhouse gas and contributor to ozone layer destruction, and its release from fixed N is almost entirely controlled by microbial activities. Mitigation of $N_2O$ emissions to the atmosphere has been attributed exclusively to denitrifiers possessing NosZ, the enzyme system catalyzing $N_2O$ to $N_2$ reduction. We demonstrate that diverse microbial taxa possess divergent *nos* clusters with genes that are related yet evolutionarily distinct from the typical *nos* genes of denitrifers. *nos* clusters with atypical *nosZ* occur in Bacteria and Archaea that denitrify (44% of genomes), do not possess other denitrification genes (56%), or perform dissimilatory nitrate reduction to ammonium (DNRA; (31%). Experiments with the DNRA soil bacterium *Anaeromyxobacter dehalogenans* demonstrated that the atypical NosZ is an effective $N_2O$ reductase, and PCR-based surveys suggested that atypical *nosZ* are abundant in terrestrial environments. Bioinformatic analyses revealed that atypical *nos* clusters possess distinctive regulatory and functional components (e.g., Sec vs. Tat secretion pathway in typical *nos*), and that previous *nosZ*-targeted PCR primers do not capture the atypical *nosZ* diversity. Collectively, our results suggest that nondenitrifying populations with a broad range of metabolisms and habitats are potentially significant contributors to $N_2O$ consumption. Apparently, a large, previously unrecognized group of environmental *nosZ* has not been accounted for, and characterizing their contributions to $N_2O$ consumption will advance understanding of the ecological controls on $N_2O$ emissions and lead to refined greenhouse gas flux models.**

nitrogen cycle | climate change

Fossil fuel combustion, agricultural practices (e.g., the cultivation of legumes promoting microbial $N_2$ fixation, manure application), and the conversion of nonreactive $N_2$ to ammonium in the Haber–Bosch process are the main causes of the increased input of fixed (reactive) nitrogen (N) into the environment (1). This anthropogenic contribution to the N imbalance has a series of environmental consequences, and particularly troubling are climate change concerns stemming from the release of $N_2O$ gas into the atmosphere. $N_2O$ is a greenhouse gas with a global warming potential 310 times greater than that of the equivalent amount of $CO_2$ (2–4) and promotes ozone destruction in the stratosphere (5–8). Global measurements demonstrate that atmospheric $N_2O$ has increased steadily over the past 250 y (9), indicating that current global sources exceed global sinks [(10); World Meteorological Organization, www.wmo.int/pages/mediacentre/press_releases/documents/GHG_bull_6_en.pdf]. The major sources for atmospheric $N_2O$ are biotic and coupled abiotic processes occurring in soil, sediment, and subsurface ecosystems (11–14). Diverse microbial populations of complete denitrifiers ($NO_3^-/NO_2^- \rightarrow N_2$), incomplete denitrifiers ($NO_3^-/NO_2^- \rightarrow N_2O$), nitrate reducers ($NO_3^- \rightarrow NO_2^-$), ammonifiers [i.e., microorganisms performing dissimilatory nitrate reduction to ammonium (DNRA)] ($NO_3^-/NO_2^- \rightarrow NH_4^+$), and nitrosifyers ($NH_4^+ \rightarrow NO_2^-$) contribute to $N_2O$ release (15–21), and additional $N_2O$ emissions result from coupled microbial–abiotic processes (chemodenitrification). For example, ferric iron-reducing bacteria generate ferrous iron, which reacts chemically with nitrite to produce $N_2O$ (22–24). Contemporary greenhouse gas models presume that $N_2O$-to-$N_2$ reduction (i.e., the final step of the denitrification pathway) is the major attenuation process controlling $N_2O$ flux to the atmosphere. Denitrification *sensu stricto* (i.e., complete denitrification) is a process catalyzed by microorganisms that possess the enzymatic machinery including Nar and/or Nap ($NO_3^- \rightarrow NO_2^-$), Nir ($NO_2^- \rightarrow NO$), Nor ($NO \rightarrow N_2O$), and Nos ($N_2O \rightarrow N_2$) for the stepwise reduction of $NO_3^-$ to $N_2$ (21). Attempts to predict $N_2O$ emissions based on denitrifier *nosZ* gene abundance and expression revealed an incongruity between the predicted and the actual $N_2O$ emissions, suggesting the existence of an unaccounted $N_2O$ sink (25–28). To explore the basis of this discrepancy, we screened 126 bacterial and 7 archaeal genomes containing *nosZ* and found a broad distribution of *nosZ* genes across 16 taxonomic groups of Bacteria and the Archaea. This analysis revealed uncharacterized (novel) *nosZ* genes encoding functional NosZ ($N_2O$ reduction to $N_2$) in diverse taxonomic groups, far exceeding the known *nosZ* diversity of complete denitrifiers. Thus, our study expands the current understanding of the nitrogen cycle and provides enhanced means to monitor and model $N_2O$ emissions into the atmosphere.

## Results and Discussion

**Expanded NosZ Diversity and Prevalence in Taxonomically Diverse Populations.** To date, $N_2O$-to-$N_2$ reduction in the environment has been attributed exclusively to denitrifying microorganisms expressing the typical Z-type NosZ (29, 30). Typical *nosZ* genes (77 sequences) were found on 75 genomes belonging to the Alpha-, Beta-, and Gammaproteobacteria harboring complete sets of denitrification genes. Ten genomes harboring a typical *nosZ* lacked *nirS* or *nirK* homologs, corroborating previous observations that some microorganisms with a typical *nosZ* may not denitrify (21, 30). Interestingly, bioinformatic sequence analyses

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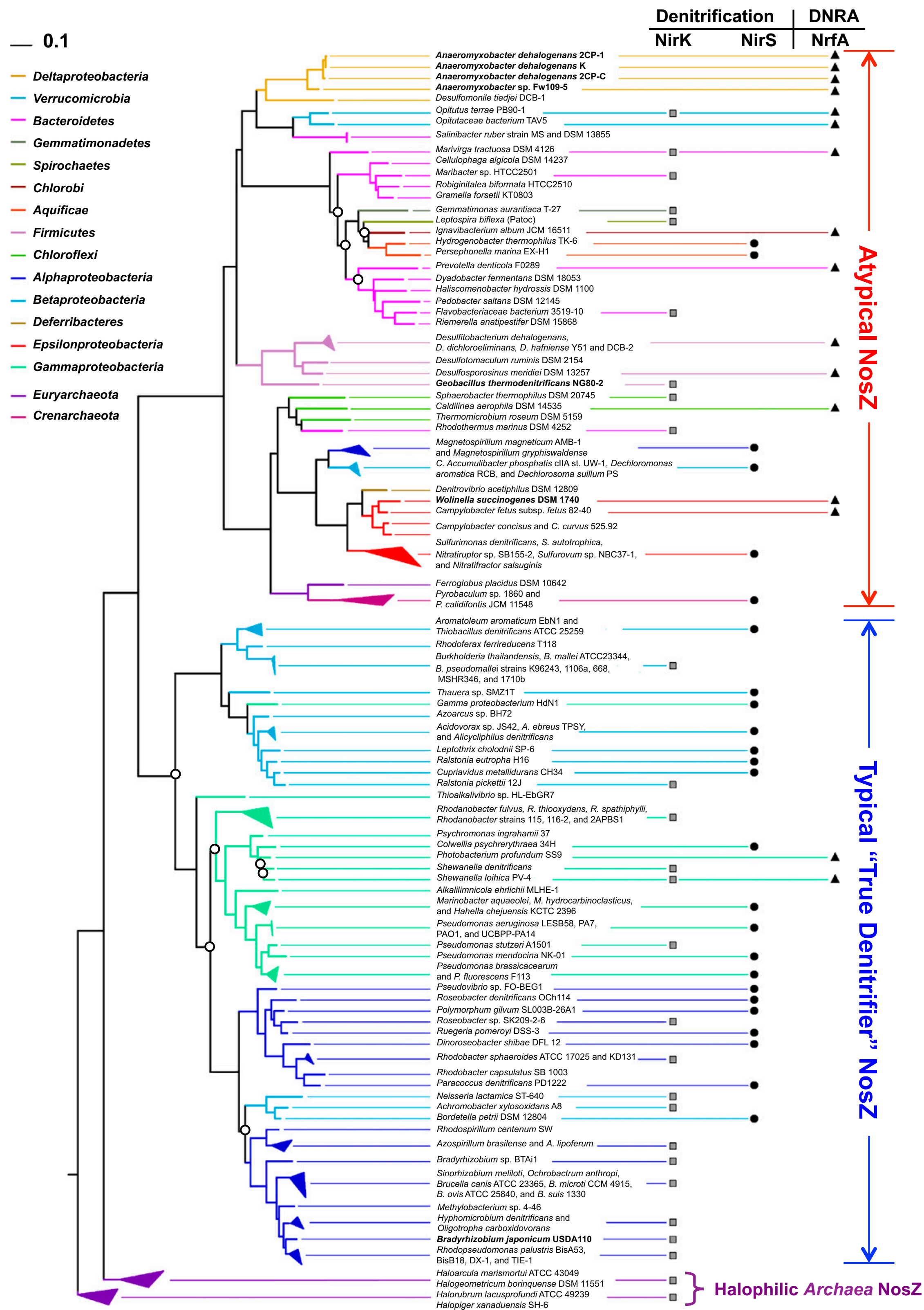


**Fig. 1.** Bootstrapped neighbor-joining phylogeny of 136 NosZ sequences representing 133 genomes. Branches with bootstrap support <50% are indicated by open circles. The typical NosZ clade is composed of sequences with an N-terminal Tat motif and associated primarily with functionally characterized soil denitrifiers of the *Proteobacteria*. The atypical NosZ is distributed among Bacteria and Archaea, and has been characterized in *A. dehalogenans*, *W. succinogenes*, and *G. thermodenitrificans* (bold font). *nosZ* occurs as a single-copy gene on all genomes, except for *Pseudomonas brassicacearum* and *Sulfurimonas denitrificans*, which both have two *nosZ* loci. Also indicated is the presence of nitrite respiratory genes *nirS*/*nirK* (denitrification) and *nrfA* (DNRA ammonification). Four NosZ within the halophilic Euryarchaeota formed a discrete cluster and did not group with either the atypical or the typical NosZ. The scale bar at the top left corresponds to the mean number of amino acid substitutions per site.



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identified bacteria with phylogenetically distinct, atypical *nosZ* genes in diverse microbial taxa abundant in terrestrial and marine environments. The NosZ phylogeny in Fig. 1 displays the broad diversity of organisms with regard to both taxonomic affiliation and predicted *N*-respiration metabolism. Of 136 NosZ protein sequences, 77 represented the typical NosZ encoded on the genomes of well-characterized complete denitrifiers (e.g., *Bradyrhizobium japonicum*). A separate cluster with robust bootstrapping support comprised 55 atypical NosZ sequences, which were distributed across genomes of 13 bacterial and 2 archaeal phyla (Fig. 1). Fewer than half of these atypical NosZ (i.e., 24 sequences) resided on genomes carrying *nirK* or *nirS*, which are hallmark genes of the denitrification process. Remarkably, atypical *nosZ* genes were found on 15 genomes possessing *nrfA* but lacking *nirK* or *nirS*, on 16 genomes lacking any known type of nitrite reductase (i.e., not possessing *nrf*, *nor*, or *nir* genes), and 2 genomes with both *nrf* and *nir* genes. The latter observation reveals that complete denitrification and ammonification pathways may be encoded on the same genome, contrasting with the prevailing view of pathway incompatibility (31). Both pathways coexist in the genomes of *Opitutus terrae* and *Marivirga tractuosa* possessing atypical *nosZ* genes, and in *Shewanella loihica* with a typical *nosZ* gene, indicating that some organisms are capable of nitrate reduction to both ammonium and gaseous products. Importantly, these analyses demonstrate that taxonomically diverse microbes possess atypical *nosZ* genes, including the thermophilic Aquificae and the deep-branching Chloroflexi phyla (Fig. 1), indicating that $N_2O$-to-$N_2$ reduction in soils, sediments, and other habitats is not limited to the activity of complete denitrifiers harboring the typical *nosZ*.

**Evidence That Atypical *nosZ* Genes Encode $N_2O$ Reductases.** Observations with the eukaryotic host-associated bacterial species *Wolinella succinogenes* and *Campylobacter fetus* provided the first evidence that the atypical NosZ are functional, as both these species cannot denitrify but grow with $N_2O$ as electron acceptor (32, 33). Subsequent biochemical and genetic studies corroborated functionality as $N_2O$ reductases; however, the diversity and abundance of atypical *nosZ* were not explored (Fig. 1). Of environmental significance are *Anaeromyxobacter* spp., which are distributed in many soil ecosystems based on their detection in and/or isolation from arid, forest, tropical, and compost soils (34–36). The atypical NosZ of *Anaeromyxobacter dehalogenans* shares only 33% amino acid identity with the typical Z-type NosZ of the well-characterized denitrifier *B. japonicum* (37, 38). To verify the physiological function of this atypical NosZ as an $N_2O$ reductase, growth experiments with the nondenitrifying species *A. dehalogenans* were conducted. Consistent with the lack of *nir* genes associated with the denitrification pathway and the presence of *nrfA*, *Anaeromyxobacter* strains reduced nitrate and nitrite stoichiometrically to ammonium (*SI Appendix*, Fig. S1) (35). Growth also occurred with $N_2O$ as electron acceptor (Fig. 2), indicating that the atypical *Anaeromyxobacter*-type *nosZ* does encode a respiratory terminal $N_2O$ reductase, which is consistent with NosZ activity observed in *Wolinella*, *Campylobacter*, and *Geobacillus* (32, 33, 39, 40). Acetate-fed *A. dehalogenans* strain 2CP-C cultures produced 2.85 ± 0.44 and 4.08 ± 1.22 × $10^8$ cells per micromole of $N_2O$ reduced, demonstrating that $N_2O$ reduction was linked to energy conservation (*SI Appendix*, Table S1). Compared with the true denitrifier *Pseudomonas stutzeri* strain DCP-Ps1, the cell yield of strain 2CP-C was about 1.5-fold higher [6.4 mg (dry weight) vs. 4.3 mg/mmol $N_2O$], indicating that the *Anaeromyxobacter* NosZ enzyme is involved in energy conservation and may operate with greater efficiency than the respiratory machinery of the complete denitrifier. It also is well documented that complete denitrifiers exhibit inhibition of $N_2O$ respiration in the presence of nitrate (41, 42), but *Anaeromyxobacter* cultures amended with 1 mM nitrate did not show diminished $N_2O$ reduction rates. Consistent with these physiological observations,

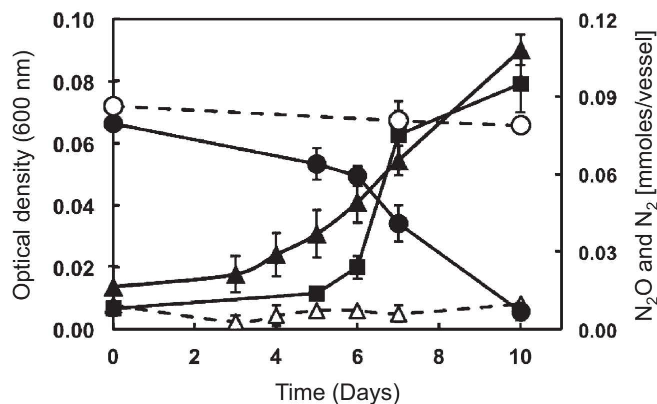


**Fig. 2.** Growth of *A. dehalogenans* strain 2CP-C with $N_2O$ as the sole electron acceptor. The medium was amended with acetate as electron donor, and the vessels were inoculated with acetate/$N_2O$-grown 2CP-C cells that had consumed all $N_2O$. The data points represent the averages of triplicate cultures, with error bars showing the SD. In control vessels without $N_2O$, cell numbers did not increase. No $N_2O$ reduction occurred in control cultures that received no inoculum. ●, $N_2O$; ■, dinitrogen; ▲, cells = $OD_{600\ nm}$; ○, $N_2O$, no cells; △, cells = $OD_{600\ nm}$, no $N_2O$.

the bioinformatics analysis revealed distinct features of the *nos* cluster with atypical NosZ (see below).

A genetic system for *Anaeromyxobacter* is not available to elucidate the detailed function(s) of individual components of the atypical *nos* cluster; however, multiple independent lines of evidence support that the atypical NosZ catalyzes $N_2O$ reduction. Querying sequences of typical *nosZ* sequences against the available strain 2CP-C genome revealed that only Adeh_2402 (ABC82172), the gene encoding the atypical NosZ, yielded a robust match. In fact, only the atypical NosZ, but no other oxidoreductases or cytochromes encoded on the strain 2CP-C genome, shared the key characteristic amino acid residues (*SI Appendix*, Fig. S2). The atypical *nosZ* also is part of a *nos* operon, which does not appear to be missing any key functional component compared with typical *nos* clusters (Fig. 3). Finally, the atypical NosZ of *Geobacillus thermodenitrificans* and the nondenitrifying rumen symbiont *W. succinogenes* have been demonstrated to reduce $N_2O$ (33, 40) and show relatively high amino acid identity to the *A. dehalogenans* NosZ (39% and 53%, respectively), indicating that the latter homolog encodes the key $N_2O$ reductase on the *A. dehalogenans* genomes.

Mature NosZ are located in the periplasm or are membrane associated (30), and the typical and atypical NosZ differ in regard to the secretion pathway used to deliver the protein across the cytoplasmic membrane. All typical NosZ possess the twin-arginine translocation (Tat) signal peptide with the characteristic [RRx(F|L)] motif (43). In contrast, the atypical NosZ, except those of the *Chloroflexi*, possess an N-terminal Sec-type signal peptide (44). Additional differences between typical and atypical NosZ proteins occur in two of the seven conserved histidine residues involved in binding of the catalytic copper center ($Cu_Z$) (30, 45). Among the 77 typical NosZ, the $Cu_Z$-binding motifs associated with the first two histidines, DxHHxH, and the last histidine, EPHD, exhibited 100% conservation (*SI Appendix*, Fig. S2). Fewer conserved residues were found in the $Cu_Z$-binding motifs of atypical NosZ (i.e., DxHH and EPH), suggesting structural and possibly mechanistic differences that may explain the increased growth efficiency observed in *A. dehalogenans* strain 2CP-C.

**Genomic Context of *nosZ* Genes in Nondenitrifying Populations.** The genomic loci encoding NosZ are part of the *nos* cluster, which includes genes encoding accessory proteins required for NosZ maturation and function. The *Anaeromyxobacter nos* clusters

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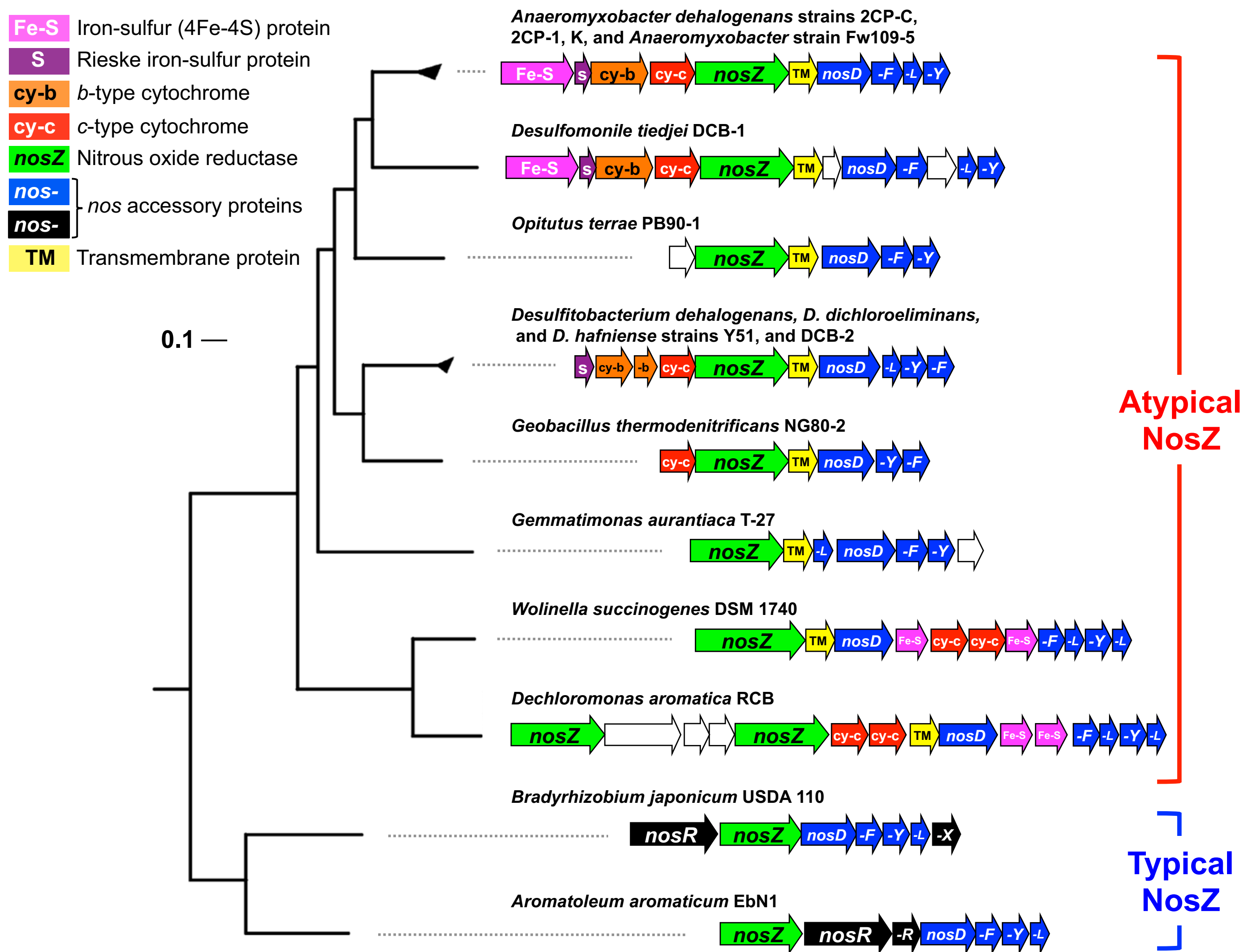


**Fig. 3.** Comparison of *nos* clusters carrying the atypical *nosZ* with the characterized *nos* cluster of the complete denitrifier *B. japonicum* strain USDA110, which harbors a typical *nosZ*. *nos* clusters of *Anaeromyxobacter* sp. strain Fw109-5, three *A. dehalogenans* strains, *O. terrae*, *D. tiedjei*, *G. thermodenitrificans*, four representatives of the *Desulfitobacterium* genus, *G. aurantiaca*, *W. succinogenes*, and *Dechloromonas aromatica* harbor the atypical *nosZ* and encode predicted iron–sulfur-binding proteins (labeled "4Fe-4S" or "Fe-S"), Rieske iron–sulfur proteins (S), *b*-type cytochromes (cy-b), or *c*-type cytochromes (cy-c). The gene encoding a protein with up to four predicted transmembrane-spanning regions (TM) is found in all atypical, but none of the typical, *nos* clusters. Accessory genes (*nosD*, *nosF*, *nosL*, and *nosY*) are generally conserved across *nos* clusters with both typical and atypical *nosZ*. Noncolored genes in the *A. dehalogenans* and *O. terrae* operons have no orthologs in any other known *nos* cluster. *nosR* and *nosX* are associated exclusively with typical *nos* clusters. *W. succinogenes* produces a unique NosZ with an additional C-terminal c-type cytochrome domain. The scale bar indicates the mean number of amino acid substitutions per site.

comprise 10 or 11 genes and share the 4 NosZ maturation genes *nosD*, *nosF*, *nosY*, and *nosL* with the denitrifier-characteristic *nos* clusters, comprising only 6–8 genes (Fig. 3). All *nos* clusters with the atypical *nosZ* lack *nosR* and *nosX*, which have been implicated in NosZ expression (37, 46, 47) and electron transfer to NosZ (30), respectively, in complete denitrifiers. Distinctive features of the *Anaeromyxobacter nos* clusters included four genes upstream of *nosZ*, which encode proteins with two 4Fe-4S iron–sulfur motifs (**CxxCxxCxxxCP**), a 2Fe-2S Rieske iron–sulfur motif (**CxH**. . .**CPCH**), two *b*-type cytochrome domains [pfam0003(2|3)], and five *c*-type cytochrome (**CxxCH**) motifs, respectively, suggesting they serve electron transport functions (Fig. 3). Structurally similar *nos* clusters were encountered in *Desulfitobacterium* spp. and *Desulfomonile tiedjei*; however, the presence and organization of genes encoding electron transfer proteins were variable in other *nos* clusters with atypical *nosZ*. For example, the *nos* cluster of the Epsilonproteobacterium *W. succinogenes*, a bovine rumen symbiont (33), produces a NosZ with a *c*-type cytochrome domain near the C terminus, and genes with electron transfer functions are located downstream of *nosZ*. Another unique feature of all *nos* clusters with the atypical *nosZ* was a gene encoding a transmembrane protein of unknown function typically located immediately downstream of *nosZ* (Fig. 3).

**Current PCR Primers Underestimate *nosZ* Abundance and Activity.** Alignment of multiple *nosZ* sequences that included both typical Z-type and atypical *nosZ* genes revealed sequence divergence (i.e., mismatches) at sites targeted by previously designed *nosZ*-specific PCR primers that had been used to estimate the abundance and activity of complete denitrifiers (48–53) (*SI Appendix*, Fig. S3). PCR assays confirmed that commonly used *nosZ*-targeted primer sets including Nos661F/Nos1773R (52), NosZ-F/nosZ1622R (51, 53), nosZ1F/nosZ1R, and nosZ2F/nosZ2R (49) are not comprehensive and fail to amplify the atypical *nosZ* genes. Conserved sequence features distinguished the atypical and typical *nosZ* genes and are suitable for designing PCR primer sets that selectively amplify atypical *nosZ* genes. Based on the *nosZ* sequences from five available *Anaeromyxobacter* genomes, we designed primers NosZ-912F



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and NosZ-1853R to amplify an ∼880 bp–long *nosZ* fragment (*SI Appendix*, Table S2). This primer pair, designed to target the *Anaeromyxobacter nosZ*, did not amplify the typical *nosZ* of *P. stutzeri* or the atypical *nosZ* of *Desulfitobacterium* spp. and *W. succinogenes*.

**Environmental Distribution of *Anaeromyxobacter* spp. with Atypical *nosZ*.** To assess the prevalence of *Anaeromyxobacter* in natural environments, we collected 145 soil samples from different agricultural sites located in the Illinois Corn Belt. Using an *Anaeromyxobacter* 16S rRNA gene-targeted, genus-specific primer set (36), we found that 140 of these samples tested positive, indicating the common distribution of *Anaeromyxobacter* spp. across a range of texturally distinct agricultural soils (*SI Appendix*, Fig. S4). Further analysis performed with 77 soil samples collected from two Illinois field sites (Dekalb and Havana) with contrasting soil textures and drainage characteristics demonstrated the abundance of *Anaeromyxobacter* 16S rRNA genes along with the presence of the atypical *Anaeromyxobacter*-type *nosZ* gene. Between $1 \times 10^5$ and $3 \times 10^7$ *Anaeromyxobacter* cells per gram of soil were enumerated using quantitative real-time PCR (qPCR), and the *Anaeromyxobacter*-type *nosZ* gene was detected using direct and nested PCR in 62 (81%) of the agricultural soil samples (*SI Appendix*, Fig. S5). The abundance of *Anaeromyxobacter* 16S rRNA genes showed a statistically significant correlation with the presence of the atypical *Anaeromyxobacter*-type *nosZ* gene (Fig. 4), corroborating that the direct/nested PCR specifically amplified *Anaeromyxobacter*-type *nosZ* gene fragments, and supporting the hypothesis that nondenitrifiers with the atypical *nosZ* represent an important and heretofore unrecognized group contributing to $N_2O$ consumption in soils. PCR amplicons obtained from three Illinois soils (Havana, Urbana, and Dekalb) with *A. dehalogenans*-specific *nosZ* primers were cloned and sequenced. Amino acid sequences inferred from 11 distinct *nosZ* fragments (∼900 bp) shared high similarity with NosZ of *A. dehalogenans* strain 2CP-C (*SI Appendix*, Fig. S6), ranging from 74% to 94% identity (77–91% pairwise nucleotide identity). Among the atypical *nosZ* amplicons retrieved from the three sites, nine other sequences had high similarity (>72% amino acid identity) to the NosZ identified in *Gemmatimonas aurantiaca*, suggesting a wider range of atypical *nosZ* homologs is present in these soils. The abundance of complete denitrifiers in soils ranges from $10^5$ to $10^7$ cells per gram (49), similar to the numbers of *Anaeromyxobacter* cells we quantified in the agricultural soils. Considering that *nos* clusters with atypical *nosZ* genes occur in nondenitrifying (and some denitrifying) genera common to soil ecosystems, and that our primers targeted only a subset of the atypical *nosZ* genes, the relative abundance of atypical NosZ may in fact exceed that of typical Z-type NosZ. In support of this hypothesis, additional common soil bacteria, including ammonifying *Desulfitobacterium* spp. and denitrifying *Bacillus, Geobacillus,* and *Dechloromonas* spp., also harbor atypical *nosZ* genes (Fig. 1) (50).

**Conclusions and Future Perspectives.** All complete denitrifiers are facultative aerobes and represent an ecophysiologically homogeneous group that switches from oxygen respiration to denitrification when soils become anoxic following rain events. The nondenitrifying $N_2O$ reducers with atypical NosZ are ecophysiologically more diverse and occupy a much broader range of habitats, including anoxic, microaerophilic, oxic, psychrophilic, piezophilic, thermophilic, and halophilic environments. These findings indicate that microbial populations with atypical *nosZ* genes, including nondenitrifying *Anaeromyxobacter* spp., are potential contributors to $N_2O$ reduction in soils and sediments, as well as in other habitats where $N_2O$ sources (e.g., chemodenitrification, nitrification) exist. These findings further demonstrate that the combined contributions of both complete denitrifier and nondenitrifier $N_2O$ reducers must be quantified to obtain a meaningful measure of the catalysts involved in $N_2O$ reduction.

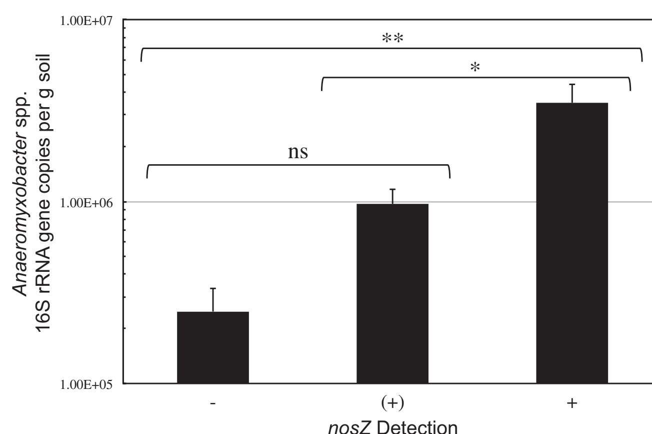


**Fig. 4.** Correlation of *Anaeromyxobacter* spp. abundance with the relative detection of atypical *Anaeromyxobacter nosZ* genes in Dekalb and Havana spatial variogram soil core samples. Mean 16S rRNA gene copy numbers per gram of soil are plotted in each *nosZ* relative abundance group. Kruskal–Wallis one-way ANOVA demonstrated significant differences between negative (–, no *nosZ* amplicons obtained) and positive (+, *nosZ* amplicons obtained in direct PCR) PCR results (**$P < 0.0001$), and between positive and low target gene abundance [(+), *nosZ* amplicons obtained with nested PCR] results (*$P < 0.05$). No significant statistical relationship (ns) was observed between the abundance of detected *Anaeromyxobacter* populations and samples that tested negative for *nosZ* or required nested PCR for *nosZ* detection. Error bars represent the SD.

Because the currently applied molecular tools used to estimate *nosZ* gene activity are not comprehensive and miss the contributions of microbes carrying an atypical *nosZ* gene, current assessments of *nosZ* transcripts would underestimate the actual activity. The environmental *nosZ* sequences reported here will aid the design of refined PCR tools to comprehensively assess and measure the abundance and activity of microbes controlling $N_2O$ flux.

$N_2O$ is a potent greenhouse gas that destroys the ozone layer and therefore affects global warming and climate change. Obviously, the processes that affect (i.e., control) $N_2O$ flux must be understood, and substantial efforts have elucidated $N_2O$ sources to generate refined $N_2O$ emission models (4, 54, 55). To date, complete denitrifiers have been considered the key functional guild that controls $N_2O$ emissions from soil and sediments to the atmosphere (28, 56–58). Our results imply that the analysis of the typical denitrifier *nosZ* provides an incomplete picture and is insufficient to account for or accurately predict $N_2O$ flux. The discovery of functional, atypical *nosZ* genes from Bacteria and Archaea from a variety of habitats, including agricultural soils, indicates that a much broader group of microbes contributes to $N_2O$ turnover. This heretofore unrecognized diversity broadens our understanding of the ecological controls of $N_2O$ consumption, and the contributions of microbes with atypical *nosZ* genes should be considered in monitoring regimes and future greenhouse gas flux models.

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## Materials and Methods

Details of all methods used in this study are described in the *SI Appendix, Materials and Methods*. The *SI Appendix* is divided into three sections: (*i*) organisms, culture conditions, growth yield measurements and analytical procedures; (*ii*) molecular techniques including DNA extraction, primer design and PCR protocols; and (*iii*) computational analyses and methodologies. Additional references provide further information about procedures and analytical techniques.

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